Approaches for *Mycoplasma hyopneumoniae* detection, control, and molecular characterization

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Dedication

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Abstract

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is a prevalent respiratory bacterium known to inflict significant health challenges in swine. Opportunities to improve disease control efforts exist, as the continuation or reoccurrence of *M. hyopneumoniae* infection occurs. The general objective of this thesis was to provide new information on the detection, control, and molecular characterization of this microorganism.

Insight in the detection and rate of pathogen spread is essential to guide surveillance and control efforts. Therefore, the detection and transmission of *M. hyopneumoniae* were evaluated under natural conditions. Bacterial transmission and clinical signs were not evident until several weeks after the introduction of one infected pig, highlighting the need for accurate surveillance protocols.

Despite their frequent use for disease control and elimination, there is limited information on whether vaccines and antibiotics can alter the spread and persistence of *M. hyopneumoniae*. The effect of multiple vaccinations on *M. hyopneumoniae* infection and transmission was explored. Results indicated that a three-dose vaccination strategy against *M. hyopneumoniae* numerically reduced pathogen transmission, especially when the entire population was vaccinated. Next, the effect of medication on *M. hyopneumoniae* transmission and detection by PCR was evaluated during different infection phases. Medication numerically reduced the rate of new infections during the acute phase of infection. For the chronic phase, persistence of *M. hyopneumoniae* detection by PCR was evident for several months post-treatment. However, the infectiousness (or transmission potential) of the bacterium could not be assessed due to

lack of transmission. Further research focused on the effect of control measures on *M. hyopneumoniae* transmission is warranted.

Multiple-Locus Variable number tandem repeat Analysis (MLVA) and complete P146 gene sequencing were employed to characterize *M. hyopneumoniae* variants. Fair agreement in assay outcome was calculated between the two methods. Discriminatory power was higher for MLVA than the P146 sequencing. Nevertheless, similar epidemiological inferences were obtained. Using MLVA, genetic diversity of *M. hyopneumoniae* was evaluated across swine production flows. Insight for pathogen origin was provided based on the presence of common *M. hyopneumoniae* variants between finishers and the sourcing herds.

In conclusion, results from this thesis highlight the importance of diagnostics and epidemiology of *M. hyopneumoniae* for overall disease control.

Table of Contents

List of Tables

List of Figures

General Introduction

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is one of the most prevalent bacterial agents to cause respiratory disease in pigs worldwide and is a primary pathogen in the Porcine Respiratory Disease Complex (Pieters and Maes, 2019). Infections with *M. hyopneumoniae* affect herd health and productivity due to the development of a dry chronic cough and reduction in growth parameters, which often persists until marketing and results in significant economic losses. Moreover, *M. hyopneumoniae* infections predispose pigs to secondary bacterial and viral co-infections through the impairment of primary host respiratory defense mechanisms (Debey et al., 1992; Ross 1992), thus exacerbating disease challenges and increasing economic losses. The economic impact of *M. hyopneumoniae* has been estimated to be $3.53-7.00 per marketed pig, which can equate to $877,375 for a 5,000 breed-to-finish operation per year (Haden et al., 2012; Silva et al., 2019).

Mycoplasma hyopneumoniae is known to spread into susceptible populations via the introduction of infected pigs and infectious aerosol particles (Pieters and Maes, 2019). The role of contaminated fomites or personnel in the spread of *M. hyopneumoniae* remains unclear, however, it is generally thought to be minor due to short pathogen survival times in the environment (Goodwin, 1985; Batista et al., 2004; Browne et al., 2017). Once *M. hyopneumoniae* has been established in a herd, endemicity of infection is thought to occur through the presence of susceptible subpopulations and their contact with respiratory secretions from infected pigs (Fano et al., 2005). Challenges with achieving pathogen control are mainly attributed to the slow and persistent nature of *M. hyopneumoniae* infection.

Several control measures, like vaccination and medication, are implemented to lessen disease severity (Maes et al., 2008), however, little is known about the effect of control strategies on *M. hyopneumoniae* transmission. In recent years, efforts have been made to employ long-term measures of pathogen control, namely *M. hyopneumoniae* elimination strategies (Holst et al., 2015), followed by robust biosecurity practices and surveillance protocols. However, *M. hyopneumoniae* outbreaks still occur, thus questioning the potential source of pathogen introduction and the efficacy of control, elimination, and prevention strategies.

To aid in *M. hyopneumoniae* control and elimination efforts, this thesis aims to provide new information on the detection, control, and molecular characterization of this microorganism. Therefore, the following specific objectives were proposed: a) evaluate the detection and transmission of *M. hyopneumoniae* following natural infections; b) assess the effect of control interventions on pathogen detection and transmission; c) characterize and compare *M. hyopneumoniae* variants using different molecular techniques to aid future epidemiological investigations and pathogen tracing; and d) evaluate genetic diversity of *M. hyopneumoniae* within production flows to provide insight on inter-herd transmission.

To optimize control and elimination efforts, detecting and accurately measuring the rate at which the pathogen can spread in a population is critical, especially under conditions that can be extrapolated to the field. From this, sampling protocols can be developed to either confirm freedom of disease in a naïve population or surveil for new infections. As of today, our knowledge of *M. hyopneumoniae* transmissibility has been based on data generated from clinical samples of limited diagnostic sensitivity (Pieters et

al., 2017) under experimental conditions (Meyns et al., 2004; Villarreal et al., 2011a). In Chapter 2, the natural transmission and detection of *M. hyopneumoniae* were evaluated to estimate the rate of pathogen spread in a naïve population and to explore the likelihood of missing a recent introduction based on clinical samples with high diagnostic sensitivity.

Improvements in understanding whether control interventions can influence the degree of *M. hyopneumoniae* transmission are warranted. Currently, there is minimal information on whether vaccines and antibiotics can affect *M. hyopneumoniae* transmission, and to what extent this occurs. Therefore, opportunities to use control interventions in a strategic and effective manner to aid in the reduction *M. hyopneumoniae* transmission in a population exist. In Chapter 3, the effect of multiple vaccinations on *M. hyopneumoniae* infection and transmission rate was assessed using an *in vivo* seeder-to-contact model. Moreover, Chapter 4 explored differences in *M. hyopneumoniae* detection and transmission when acutely and chronically infected pigs were treated with a bacteriostatic antibiotic commonly used for the therapeutic and metaphylactic treatment of *M. hyopneumoniae* infection.

Mycoplasma hyopneumoniae control and biosecurity measures at the herd or regional level are supported by the ability to detect and trace pathogen spread. Over the years, there has been a growing need to utilize molecular characterization techniques in the field to better understand the inter-herd transmission of *M. hyopneumoniae* or even distinguish between elimination failures or new disease introductions.

In the past decade, the swine industry has shifted its focus to control *M. hyopneumoniae* infection at the sow herd level to influence disease outcome in downstream flows proactively, as infected sow herds are considered the reservoir for

pathogen origin for downstream grower-finisher pigs. This perception has stemmed from our current knowledge in the epidemiology of this bacterium. Research aimed towards the application of molecular techniques to epidemiologically link *M. hyopneumoniae* infection between herds is necessary to support this hypothesis. However, there is limited knowledge of the interpretation and comparison of molecular characterization techniques for *M. hyopneumoniae*, along with the epidemiological insight gained from the genomic analyses. In the United States, there are two commonly employed molecular methods, namely, Multiple-Locus Variable number tandem repeat Analysis (MLVA) and the complete gene sequencing of the adhesin-like protein, P146. Nevertheless, questions surrounding the discriminatory power of these molecular techniques and their ability to infer the genetic relatedness between variants currently exists. In Chapter 5, circulating *M. hyopneumoniae* variants in the Midwestern United States were characterized and compared using the two commonly employed molecular methods. Based on the findings from Chapter 5, MLVA was employed in Chapter 6 to evaluate the genetic diversity of *M. hyopneumoniae* within production flows.

New knowledge gained from this thesis will allow veterinarians to make informed decisions that will improve surveillance, control, and elimination efforts for *M. hyopneumoniae.*

Chapter 1: Literature review

Partial work from this chapter has been published in:

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1.1 Mycoplasmas

Mycoplasmas are a genus of bacteria classified under the class *Mollicutes* and family *Mycoplasmataceae*, and are known to colonize and affect several species of plants, insects, and animals (Razin et al., 1983). Mycoplasmas are recognized as the smallest, self-replicating prokaryotes, ranging from 0.2 to 0.8 μm in diameter (Razin et al., 1983). Morphologically, Mycoplasma colonies are pleomorphic and resemble a "fried egg" in agar, consisting of a central, granular zone embedded in agar and a flat peripheral zone on the surface (Razin et al., 1983). Most Mycoplasmas are non-motile; however, some species exhibit structures that allow for movement, adhesion, and even penetration of host cells (Razin and Jacobs, 1992).

Through phylogenetic analyses, it has been suggested that *Mollicutes* have evolved by degenerative evolution from Gram-positive eubacteria, allowing for unique taxonomic characteristics (Woese et al., 1980; Maniloff, 1992). A distinguishing feature that Mycoplasmas exhibit is their lack of a cell wall, making them sensitive to lysis and resistant to certain antibiotics, specifically β-lactams. Moreover, Mycoplasmas have a small genome (580 to 1380 kbp) that is composed of low guanine and cytosine (G+C) content (24-41 mol%) and utilize TGA (UGA) as a tryptophan codon rather than a stop codon (Razin, 1978; Woese et al., 1980). Due to their small genetic composition, Mycoplasmas have limited biosynthetic abilities and rely heavily on host nutrients for growth (Razin et al., 1998).

1.2 Swine Mycoplasmas

In general, Mycoplasmas have been described to be host-specific, commensal and/or pathogenic bacteria. For swine, six host-specific Mycoplasmas have been isolated,

namely: *Mycoplasma hyopneumoniae* (*M. hyopneumoniae;* Goodwin et al., 1965; Maré and Switzer, 1965), *M. hyorhinis* (Switzer, 1955), *M. hyosynoviae* (Ross and Karmon, 1970), *M. suis* (formerly *Eperythrozoon suis*; Splitter, 1950), *M. flocculare* (Meyling and Friis, 1972) and *M. hyopharyngis* (Erickson et al., 1986). Among these isolated Mycoplasmas, *M. hyopneumoniae*, *M. hyorhinis, M. hyosynoviae*, and *M. suis* are recognized as pathogens of significant importance, while *M. flocculare* and *M. hyopharyngis* have been described to be either non-pathogenic or have a limited role in disease. Pathogen classification and associated diseases for common swine Mycoplasmas are provided in Table 1.1.

1.3 *Mycoplasma hyopneumoniae*

Mycoplasma hyopneumoniae is the etiologic agent of enzootic pneumonia in swine (Goodwin et al., 1965; Maré and Switzer, 1965) and causes a dry cough and reduced weight gain in growing pigs. Early descriptions of a pig respiratory disease were reported in Germany during the 1930s and named 'Ferkelgrippe' (i.e., 'Piglet flu' in German; Köbe, 1933). However, this disease was described to be different from swine Influenza due to the presence of chronic and persistent pneumonia that lasted for several weeks, causing a reduction in growth rate and significant, cumulative economic losses (Blakemore and Gledhill, 1941; Fulton et al., 1953; Hjärre et al., 1952; Plowright, 1953; Rislakki, 1953; Goodwin and Whittlestone, 1963). The disease was later denominated as 'infectious pneumonia,' 'parainfluenza,' or 'virus pneumonia of pigs' due to the inability to isolate Influenza and the slower onset of disease (Gulrajani and Beveridge, 1951; Betts and Beveridge, 1952). At the time, the etiologic agent was misperceived to be a virus as the agent could pass through a biological filter, despite its susceptibility to antibiotics

(Betts and Beveridge, 1952). Thirty years later, Maré and Switzer (1965) in the United States, followed by Goodwin et al. (1965) in the United Kingdom published the isolation of this microorganism in solid medium and their ability to reproduce the disease in pigs, thus fulfilling Koch's postulates. They named the microorganism *'M. hyopneumoniae*' and '*M. suipneumoniae*', respectively. Shortly after, both microorganisms were shown to be serologically indistinguishable by growth-inhibition and metabolic-inhibition tests (Goodwin et al., 1967; Hodges and Betts, 1969; Takatori, 1970). From then on, the former name, *M. hyopneumoniae*, took precedence.

1.3.1 Pathogenesis

While the pathogenesis of *M. hyopneumoniae* is complex and not yet fully understood, disease development is hallmarked by bacterial migration and adherence to ciliated respiratory epithelium, impairment of mucociliary apparatus, and modulation of innate and adaptive immune responses (Debey et al., 1992; Blanchard et al., 1992; reviewed by Pieters and Maes, 2019).

Colonization with *M. hyopneumoniae* occurs through the inhalation of bacteria-containing aerosol droplets, followed by the pathogen's ability to withstand clearance from innate immune defense mechanisms, such as the mucociliary apparatus and coughing. After inspiration, *M. hyopneumoniae* penetrates the mucosal layer and adheres to ciliated epithelial cells in the trachea, bronchi, and bronchioles. Adherence is mediated through receptor-ligand interactions and surface adhesin proteins (Ross, 1999). Most surface proteins are believed to be anchored in the cytoplasmic membrane by extracellular milieu and acyl groups (Browning et al., 2011). Cilium adherence has been proposed to primarily involve a 97-kDa protein, named P97 (Hsu et al., 1997). Several

paralogs of P97, including P146 (mhp 684), P216, P116, and P135, have been involved in cilium binding and can undergo posttranslational cleavage (Tacchi et al., 2016; Kuhnert and Jores, 2020). Cleaved fragments can bind heparin, plasminogen, and fibronectin and facilitate plasmin production, which has been proposed to aid in tissue invasion and inflammation (Burnett et al., 2006; Deutscher et al., 2010, Seymour et al., 2010, Seymour et al., 2011, Bogema et al., 2012). Following *M. hyopneumoniae* adherence, ciliostasis, deciliation of respiratory epithelium, and depletion of goblet cells occur, resulting in the impairment of the mucociliary apparatus (DeBey et al., 1992). Damage of this immune defense mechanism reduces the clearance of mucus, debris, and non-host cells, thus aiding in the invasion and propagation of secondary respiratory pathogens.

Recognition of *M. hyopneumoniae* by the host involves the employment of innate pattern-recognition receptors, including Toll-like receptors 2 and 6, which identify pathogen lipoproteins and activate signaling pathways, triggering the induction of innate immune responses (Lein et al., 1999; Muneta et al., 2003; Summerfield, 2020). As a result, pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, IL-8, and tumor necrosis factor-α (TNF-α) are produced from alveolar macrophages and lymphocytes (Thacker et al., 2000; Rodriguez et al., 2004), inducing a localized inflammatory response and lymphoid hyperplasia in lungs. Moreover, the conversion of plasminogen to plasmin contributes to inflammation by stimulating macrophage signaling, resulting in reactive oxygen species and cytokine release (Syrovets et al., 2012). In turn, an influx of neutrophils and macrophages arises, followed by lymphocyte infiltration in the peribronchiolar and surrounding perivascular tissue. Several studies have proposed that *M. hyopneumoniae* infection also results in generalized immunosuppression, through the

induction of anti-inflammatory cytokines (i.e., IL-10), alteration of lymphocyte transformation, and inhibition of interferon-γ (Kishima and Ross, 1985; Thanawongnuwech and Thacker, 2004; Muneta et al., 2006; Muneta et al., 2008). Such modulatory effects of the inflammatory response and immune system are the primary drivers for enzootic pneumonia lesions.

1.3.2 Mycoplasma hyopneumoniae *infections in the swine industry*

As a primary respiratory disease, *M. hyopneumoniae* infection results in significant health and economic concerns worldwide. Economic losses are primarily attributed to increased antibiotic and vaccine usage and other resources (e.g., feed, facilities) to offset production losses for the achievement of full-value market pigs. Production losses from *M. hyopneumoniae* infection include a reduction in gain, poor feed conversion, increased body weight variation, and increased mortality and culling rates. Studies have shown a 2.8-15.9% decrease in daily weight gain, 0.6-13.8% reduction in feed conversion, and 1.3-6.0% mortality rate in endemically infected growing herds (Pointon et al., 1985; Straw et al., 1989; Silva et al., 2019; Haden et al., 2012). Moreover, Straw et al. (1990) calculated a 37.4g/day reduction in gain for every 10% of lung affected with pneumonia. Reductions in growth parameters have been described to increase the time to market by up to 30 days, resulting in increased cost per pig space and decreased number of turns per year (Noyes et al., 1990). Silva et al. (2019) also estimated an increase in antibiotic ($1.20) and vaccination ($0.25) cost per pig, resulting in $7.00 per pig marketed, when costs associated with productivity losses were included. An economic value of $0.63-$3.53 per finisher pig has been calculated for herds solely affected by *M. hyopneumoniae* and up to $10.12 per pig for herds co-

infected with *M. hyopneumoniae* and porcine reproductive and respiratory syndrome virus (PRRSv) or Influenza A virus in swine (IAV-S; Haden et al., 2012). Nevertheless, the ability to obtain accurate information related to the economic impact of *M. hyopneumoniae* infection is limited and can be challenging to ascertain, especially under field conditions, as secondary infections are often present and can also affect production and economic parameters.

Mycoplasma hyopneumoniae is a primary contributor to the Porcine Respiratory Disease Complex (PRDC). Impairment of the mucociliary apparatus and immune modulation have been identified as key underlying factors that elicit and exacerbate diseases associated with *M. hyopneumoniae* infection and secondary bacterial and viral pathogens. Studies have shown *M. hyopneumoniae* to potentiate disease severity when *Pasteurella multocida* or *Actinobacillus pleuropneumoniae* infections are present (Marois et al., 1989b; Amass et al., 1994; Marois et al., 2009; Eamens et al., 2012). Interaction of *M. hyopneumoniae* with primary viral pathogens, such as PRRSv, IAV-S, or porcine circovirus type 2 (PCV-2), often results in synergistic and/or additive effects on disease severity, depending on timing and sequence of the infections (Marois-Créhan et al., 2020). Therefore, a better understanding of the interaction between *M. hyopneumoniae* and other respiratory pathogens is essential to control enzootic pneumonia and PRDC.

Due to the health concerns and economic losses associated with enzootic pneumonia, researchers and veterinarians have strived to optimize the control of *M. hyopneumoniae* by establishing a strong understanding of pathogen-specific epidemiology, along with the development and application of accurate and effective diagnostic and treatment modalities.

1.3.3 Epidemiology

1.3.3.1 Prevalence

Mycoplasma hyopneumoniae infection has generally been thought to be distributed worldwide. Since this pathogen is non-reportable and difficult to ascertain due to clinical and diagnostic limitations, data describing *M. hyopneumoniae* prevalence in the United States or in other countries is either limited or not available. Nonetheless, *M. hyopneumoniae* infection has been historically described in almost every pig producing country (Pieters and Maes, 2019). Over the past two decades, national elimination programs for *M. hyopneumoniae* have been conducted in commercial herds and deemed successful in Switzerland (Stärk et al., 2007), Norway (Guilliksen et al., 2019), and Finland (Rautiainen et al., 2001). In the United States, results generated from a national study showed a high prevalence of *M. hyopneumoniae* in 2012, as 39% and 59% surveyed breeding and grow-finish herds (n=2,119), respectively, had reported disease challenges attributed to the infection (NAHMS, 2012). Nevertheless, more information regarding *M. hyopneumoniae* prevalence in the United States is warranted to aid in understanding disease burden in a geographical location and guide regional and/or national surveillance and control programs.

1.3.3.2 Transmission routes

Horizontal transmission is considered the main route for *M. hyopneumoniae* infection and occurs via direct contact with respiratory secretions between infected and susceptible pigs, regardless of age (Etheridge et al., 1979; Morris et al., 1995). Piglets are considered free of *M. hyopneumoniae* at birth as *in-utero* transmission has not been described, but can be colonized as early as one week of age (Sibila et al., 2007b).

Therefore, the transmission of *M. hyopneumoniae* from dams to corresponding offspring has also been considered an essential contributor to piglet colonization (Calsamiglia and Pijoan, 2000a) and disease development in downstream herds (Fano et al., 2007). Indirect transmission via aerosol plays a role in the spread of *M. hyopneumoniae* at both the intra- and inter-herd level. Aerosol transmission was initially implied as earlier studies showed the risk of disease introduction to decrease as the distance from infected herds increased (Goodwin, 1985; Jorsal and Thomsen, 1988, Stärk et al., 1992). Concordantly, *M. hyopneumoniae* detection and viability were shown in air samples collected at various distances, ranging from 2.3 (Dee et al., 2009) to 9.2 km (Otake et al., 2010) from infected herds. Nevertheless, the likelihood of *M. hyopneumoniae* transmission via aerosol or local area spread has been reported to be low as Yeske et al. (2017) showed that 8% of flows within areas of high swine herd density became laterally infected with the pathogen. Although a low probability of lateral transmission was described, high consequences can arise if the site was a *M. hyopneumoniae* negative gilt developing unit or sow herd. From this study, it is important to note that *M. hyopneumoniae* lateral transmission was assessed among finisher herds, which may differ from sow herds. Spread of *M. hyopneumoniae* via contaminated fomites or personnel has been generally thought to be of little importance due to short pathogen survival times in the environment (Goodwin, 1985; Batista et al., 2004; Browne et al., 2017) and the implementation of biosecurity measures (Batista et al., 2004; Pitkin et al., 2011).

1.3.3.3 Infection dynamics

In general, *M. hyopneumoniae* infection is hallmarked by its slow and persistent nature and is often divided into two phases: acute and chronic, based on clinical

presentation. The acute phase of infection includes the incubation period and a clinical period, marked by the onset and cessation of a non-productive cough, whereas the chronic phase is characterized by an asymptomatic, carrier state (Ross, 1999; Pieters et al., 2009). The duration of *M. hyopneumoniae* infection can last up to eight months, as Pieters et al. (2009) showed that pigs infected with a moderately virulent strain (232; Minion et al., 2004) remained PCR positive and transmitted the pathogen for up to 214 days post-inoculation (dpi), followed by the absence of *M. hyopneumoniae* detection and transmission at 240 dpi. Throughout the course of infection, progression of disease processes will occur, thus influencing the timing of clinical signs and diagnostic outcomes. Moreover, *M. hyopneumoniae* strains of different virulence, ranging from avirulent, to low and high virulence, have been described (Vicca et al., 2003), which may influence the course of infection. A summarized timeline that highlights the infection dynamics with regards to a diagnosis using detection of genetic material and antibodies for *M. hyopneumoniae,* under experimental conditions, is illustrated in Figure 1.1.

Under experimental conditions, the minimum dose to induce *M. hyopneumoniae* infection has been $1x10^4$ color-changing units (CCU)/mL (Marois et al., 2010). Dynamics of *M. hyopneumoniae* infection include a latent period of approximately five days (Pieters et al., 2017) and an incubation period of 10 to 16 days (Ross, 1999). A 5-day latent period, the period between exposure and onset of infectiousness, has been suggested, as negative PCR results have been obtained at 2 dpi in various antemortem sample types (Pieters et al., 2017). The incubation period for *M. hyopneumoniae,* the time between exposure to the pathogen and onset of clinical signs, has been estimated from experimental studies and may be longer under natural conditions. During this subclinical

period, there is a high likelihood of missing a recent disease introduction due to the absence of clinical signs, resulting in unforeseen health concerns, and limitations in diagnostic sensitivity if accurate surveillance strategies are not implemented. Moreover, the transmission of *M. hyopneumoniae* before the onset of clinical signs has not been described. However, this would be important to assess to aid in surveillance and disease control efforts.

During the acute phase of infection, cough frequency and shedding have been described to peak around 28 dpi, followed by the cessation of clinical signs at six-to-eight weeks after onset (Pieters et al., 2009; Garcia-Morante et al., 2016; Roos et al., 2016). Furthermore, the adjusted basic reproductive number has been estimated to be low (R_n=0.56-1.16) under experimental and field conditions, implying that 0.56-1.16 secondary infections per infected pig can occur in a 6-week period (Meyns et al., 2004; Villarreal et al., 2011a). At the peak of shedding, Roos et al. (2016) calculated the *M. hyopneumoniae* transmission rate (β) to be 1.28 new infected gilts/gilt-week during a four-week period. It is important to mention that parameters calculated by Roos et al. (2016) were higher than previous studies, as transmission was assessed at a time when peak of shedding has been suggested. For the chronic phase of infection, epidemiological metrics to describe the contagiousness or transmissibility of *M. hyopneumoniae* have not been estimated, mainly due to the limited feasibility of conducting *in-vivo* experimental models given the long shedding duration.

In the field, the persistence of *M. hyopneumoniae* infection and presentation of clinical signs have been well documented. Clinical signs are predominately evident during the grower-finisher period. Therefore, the origin of *M. hyopneumoniae* infection in

grower-finisher herds was historically thought to occur via lateral transmission. However, a paradigm shift in the perception of *M. hyopneumoniae* infection and control was evidenced, as a new hypothesis of the epidemiology of the bacterium had been proposed (Pijoan, 2005). Considering three important epidemiological features of *M. hyopneumoniae*: 1) long shedding duration (Pieters et al., 2009); 2) slow transmission (Meyns et al., 2004), and 3) the positive association of *M. hyopneumoniae* pre-weaning prevalence with disease severity in finishing (Fano et al., 2007); the origin and severity of *M. hyopneumoniae* infection in grower-finisher herds has been suggested to be dependent upon sourcing sow herd(s) health status and stability. Such knowledge has shifted the industry's focus to control *M. hyopneumoniae* infection at the sow herd level to influence disease outcome in downstream herds proactively. To further support such hypothesis, research aimed towards developing and applying molecular techniques to characterize *M. hyopneumoniae* is warranted. In doing so, insight of pathogen origin or inter-herd transmission may be gained, which can be utilized to optimize current strategies for disease prevention and control.

1.3.3.4 Risk factors for infection

During the lactation period, dam parity (Fano et al., 2006a; Sibila et al., 2007; Boonsoongnern et al., 2012), *M. hyopneumoniae* prevalence in farrowing dams (Pieters et al., 2014), lactation length (Pieters et al., 2014), cross-fostering (Nathues et al., 2013), and temperature at the farrowing stall-level (Nathues et al., 2013) are influential factors for piglet colonization. *Mycoplasma hyopneumoniae* prevalence is higher in pigs raised from gilts and young parity sows than those from intermediate and older parity sows (Fano et al., 2006a; Sibila et al., 2007b; Boonsoongnern et al., 2012), suggesting that gilts

and young parity sows can shed the pathogen more readily than other parities. Therefore, ensuring a negative infection status for gilts at first farrowing is considered paramount to reduce *M. hyopneumoniae* transmission to piglets (Pieters and Fano, 2016). Nevertheless, Calsamiglia and Pijoan (2000a) detected *M. hyopneumoniae* in the upper respiratory tract of intermediate to older parity sows (3rd to 7th parity). Other factors such as piglet gender and piglet birth weight were not associated with *M. hyopneumoniae* colonization at weaning (Nathues et al., 2013; Pieters et al., 2014).

Differences in meteorological conditions may play a role in aerosol transmission of *M. hyopneumoniae*, as Dee et al. (2009) showed a strong association of PCR positive air samples with low sunlight levels and temperature. In contrast, other meteorological factors such as wind speed, relative humidity, and precipitation were not of significance for *M. hyopneumoniae* transmission. Conversely, Segalés et al. (2012) showed that a high precipitation rate and low temperature increased the probability of *M. hyopneumoniae* positive or seropositive pigs, respectively. It is important to note that meteorological conditions are often dependent upon topography and seasonality. There has been limited research assessing the role of topography on *M. hyopneumoniae* infection. Regarding seasonality, several studies have shown an increased probability for *M. hyopneumoniae* infections during the autumn and winter (Goodwin, 1985; Jorsal and Thomsen, 1998; Segalés et al., 2012).

Introduction and transportation of replacement gilts remain important factors for swine pathogen entry into naïve herds. For genetic and production advancement, most commercial herds maintain a 40 to 60% replacement rate per year, requiring the introduction of gilts at various frequencies year-round (Patterson and Foxcroft, 2019).

Several biosecurity practices, including an isolation period and surveillance testing, are applied with varying accuracy to ensure the negative health status of replacement gilts (Neumann and Hall, 2019). Nevertheless, there is always a risk for either introducing a new pathogen into the herd or affecting herd stability.

1.3.4 Diagnosis

Mycoplasma hyopneumoniae diagnostic methods have been used to achieve many goals that focus on pathogen surveillance, monitoring, or obtaining a conclusive diagnosis. Although clinical signs and lung lesions may lead to a tentative diagnosis, diagnostic testing is critical for pathogen identification, confirmation, and management. Depending on the goal, the diagnostic outcome can be maximized through proper candidate, sample, and assay selection.

1.3.4.1 Clinical diagnosis

The first indication of disease attributed to *M. hyopneumoniae* is the observation of a chronic, non-productive cough. However, this clinical sign is not pathognomonic, as it can be caused by environmental factors and other infectious agents (Maes et al., 2018). The onset and severity of coughing can be inconsistent and differ based on infectious dose and strain (Vicca et al., 2003; Marois et al., 2010). Additionally, subclinical infection can occur, especially in recent and chronically infected pigs, thus posing challenges to recognize, let alone diagnose the disease. Given the poor diagnostic sensitivity and specificity attributed to clinical signs, laboratory testing is required for a conclusive diagnosis.

1.3.4.2 Bacterial isolation

Isolation of *M. hyopneumoniae* remains the gold-standard technique for confirming infection (Sibila et al., 2009), despite its low diagnostic sensitivity (2.6%; Friis, 1971). Due to the microorganism's reliance on specific host nutrients for growth, isolation of *M. hyopneumoniae* is a fastidious process dependent on several factors, such as specialized medium, initial bacterial load, sample type, and presence of other bacteria (Kobisch and Friis, 1996; Friis, 1975). Nevertheless, there is interest to improve the isolation of *M. hyopneumoniae* for research purposes, establishment of antimicrobial susceptibility profiles, and improvement of vaccines. To increase the likelihood of bacterial isolation, a procedure to optimize donor selection, sample handling, and processing has been described (Anderson et al., 2016). Moreover, the use of 5% hyper-immune anti-*M. hyorhinis* serum with 500 ug/ml cycloserine (Kobisch and Friis, 1996) and kanamycin in selective medium has been suggested to suppress *M. hyorhinis* growth (Cook et al., 2016), which is a frequent competitor during *M. hyopneumoniae* isolation.

1.3.4.3 Detection of genetic material by PCR

Although bacterial isolation is considered the gold standard for *M. hyopneumoniae* diagnostics, PCR assays are frequently employed for such purposes, as this method is extremely accurate and quick to perform. Several PCR assays have been developed for the detection of *M. hyopneumoniae* in antemortem and postmortem clinical samples (Calsamiglia et al., 1999; Dubosson et al., 2004; Strait et al., 2008). In the 1990s, conventional PCR methods were the first to be developed (Harasawa et al., 1991; Artiushin et al., 1993; Stemke et al., 1994; Mattson et al., 1995; Blanchard et al., 1996; Baumeister et al., 1998), followed by nested PCRs (Calsamiglia et al., 1999; Verdin et

al., 2000; Kurth et al., 2002), to improve assay sensitivity by amplifying a targeted region of the 16S rRNA gene with the two PCR reactions. Limitations with these PCR techniques include an increased likelihood for cross-contamination between samples during sample processing and amplification, along with a qualitative outcome. Over the years, species-specific real-time PCR assays (Dubosson et al., 2004; Strait et al., 2008) have been commonly utilized for *M. hyopneumoniae* detection instead of conventional methods. This shift has primarily occurred from diagnostic advancements and technology affordability, thus aiding the ability to obtain accurate results in real-time, while allowing for high and quick throughput with less laborious procedures. Moreover, real-time PCR techniques provide a semi- or quantitative value for the amount of genetic material detected, which can be used to understand the relative bacterial load in a sample.

Real-time PCR assays are commonly employed for *M. hyopneumoniae* surveillance, monitoring, and diagnostic purposes. While PCR testing provides significant value towards guiding control and management methods, it is important to be mindful of possible pitfalls and variables that can influence assay outcome. Given that *M. hyopneumoniae* genetic material is detected, differentiation of live or dead bacteria is not feasible. Therefore, the interpretation of PCR results becomes most difficult after control or elimination strategies have been applied, as *M. hyopneumoniae* DNA has been detected post-antibiotic treatment (Painter et al., 2012), thus posing the question of present viable cells or uncleared genetic material from the respiratory tract. In addition, differences in *M. hyopneumoniae* PCR results have been described based on the material of the collection swab (Takeuti et al., 2017a), DNA extraction method (Vangroenweghe et al., 2015a), and sample type (Pieters et al., 2017; Sponheim et al., 2020).

Diagnostic sensitivity for *M. hyopneumoniae* detection by real-time PCR has been demonstrated to vary based on the type of sample collected and the infection phase. Using real-time PCR, *M. hyopneumoniae* DNA has been detected in various antemortem clinical samples, including nasal and laryngeal swabs, and tracheal secretions. Several studies have shown the collection of tracheal secretions via deep tracheal catheters/tracheo-bronchial swabs to have the highest diagnostic sensitivity for *M. hyopneumoniae* detection compared to laryngeal swabs (Sponheim et al., 2020) and nasal swabs (Kurth et al., 2002; Fablet et al., 2010; Vangroenweghe et al., 2015a) in both experimentally and naturally infected pigs. Additionally, Pieters et al., (2017) and Moiso et al. (2020) showed that laryngeal swabs were more sensitive than nasal swabs for *M. hyopneumoniae* detection under experimental and natural conditions, respectively. More recently, positive real-time PCR results have also been obtained from aggregated sample types, such as oral fluids (Pieters et al., 2017). Due to ease of collection and non-invasiveness, the use of oral fluids for pathogen surveillance and monitoring has been of growing interest (Rotolo et al., 2016). However, very low sensitivity and inconsistent detection of *M. hyopneumoniae* have been described in oral fluids, especially during the early phase of infection (Pieters et al., 2017; Hernandez-Garcia et al., 2017; Sponheim et al., 2020). *Mycoplasma hyopneumoniae* has also been detected postmortem in bronchial swabs, lung tissue, and bronchoalveolar lavage fluid, of which bronchial swabs are the most sensitive (Kurth et al., 2002; Fablet et al., 2010; Pieters et al., 2017).

1.3.4.4 Detection of antibodies

A standard method to assess the serological response to *M. hyopneumoniae* is the Enzyme-Linked Immunosorbent Assay (ELISA). Enzyme-Linked Immunosorbent Assay

is frequently used for swine diagnostics because it is a rapid and inexpensive assay. Based on the context of use, ELISAs can provide insight towards determining the presence of maternally derived or acquired antibodies. Moreover, paired sera samples can be collected to evaluate infection or vaccine compliance. Several commercially available ELISAs have been developed for *M. hyopneumoniae*-specific IgG antibody detection, and vary based on assay type (i.e., indirect or blocking) and type of antigen used (i.e., whole cell or individual proteins; Pieters and Maes, 2019). In the United States, there are two commercially available ELISA assays, namely Idexx and Oxoid (i.e., Dako), referred to as indirect (I-ELISA) and blocking (B-ELISA) ELISAs, respectively, that are commonly used. Studies have indicated similar specificity for the two assays, whereas the B-ELISA is usually identified as numerically more sensitive than the I-ELISA (Ameri-Mahabadi et al., 2005; Erlandson et al., 2005; Fano et al., 2012; Pieters et al., 2017). Therefore, B-ELISA is often used as a confirmatory test for I-ELISA when false-positive results are suspected. Sera samples have been primarily used to surveil for *M. hyopneumoniae* infection and/or establish immune status. Several studies have shown that seroconversion occurs four to six weeks post-inoculation and is a poor indicator for shedding (Pieters et al., 2009; Deegan et al., 2016; Roos et al., 2016; Pieters et al., 2017). It has been hypothesized that the delay in seroconversion may be due to a slower presentation of *M. hyopneumoniae* antigens to the host because this pathogen adheres to the ciliated respiratory epithelium and does not invade pulmonary tissue to the same extent as other pathogens (Sibila et al., 2009). Therefore, difficulties may arise for the assessment of early *M. hyopneumoniae* infections using ELISA. In addition, the accuracy of seroconversion assays can vary by vaccine product and dosage applied to hosts, cross-

reactivity with antibodies to other bacteria (e.g., *M. floccculare*), and different test procedures (Sibilia et al., 2009; Ameri-Mahabadi et al., 2005). Moreover, there is an inability to differentiation seroconversion from vaccination or natural infection using ELISA. Due to these reasons, veterinarians are often challenged by the interpretation of serological results and making inferences without vaccine history and additional diagnostics.

1.3.4.5 Antigenic assays

An immunohistochemistry (IHC) assay was developed to detect *M. hyopneumoniae* antigen in tissues (Amanfu et al., 1984). Despite the ability to correlate antigen presence with microscopic lesions, there are several drawbacks to this assay. Lung tissue is required to conduct the IHC assay, however, the opportunity to collect postmortem samples is not always an option, especially when multiple animals are recommended for a conclusive diagnosis. Moreover, a small amount of tissue is evaluated, increasing the likelihood of obtaining a false negative result. Furthermore, a highly specific monoclonal antibody is necessary to decrease the occurrence of false positives.

1.3.5 Molecular epidemiology

1.3.5.1 Genomic diversity

The *M. hyopneumoniae* genome has been fully sequenced for six strains and has been described to be composed of 892,758-964,503 base pairs and contain a low G + C content of 28.4-28.6% (Minion et al., 2004; Vasconcelos et al., 2005; Liu et al., 2011; Siqueira et al., 2013; Han et al., 2017). Also, 679-695 predicted protein-coding sequences

have been described, in which 44-62%, 14-38%, and 18-23% encode for functional, conserved, or unique hypothetical proteins, respectively (Minion et al., 2004; Vasconcelos et al., 2005; Siqueira et al., 2013). Like other Mycoplasmas, *M. hyopneumoniae* can modify gene and antigenic expression, primarily during DNA replication (Minion et al., 2004). Within many lipoprotein and adhesion encoding genes, repetitive tandem regions of DNA, known as Variable Number Tandem Repeats (VNTRs), are present and undergo phase variation, recombination, and slipped strand mispairing during replication. Thus, resulting in adherence and antigenic variation from modifications in cell surface structure (Razin et al., 1998; Rosengarten et al., 2000; Minion et al., 2000; de Castro et al., 2006). Differences in VNTR length of several adhesins and chromosomal restriction patterns have been described across *M. hyopneumoniae* strains and isolates using whole genome or individual gene sequencing (Hsu et al., 1998; Vasconcelos et al., 2005; Garza-Moreno et al., 2019), field inversion gel electrophoresis (Frey et al., 1992), and random amplified polymorphism DNA analysis (Artiushin et al., 1996).

Using various molecular techniques, *M. hyopneumoniae* genomic diversity has been summarized across different geographic and population levels (Betlach et al., 2019). From a geographical standpoint, increased genomic heterogeneity has been demonstrated across *M. hyopneumoniae* isolates and strains originating from diverse regions (i.e., different countries) compared to those originating from a more localized region (Frey et al., 1992; Dos Santos et al., 2015). Several *M. hyopneumoniae* VNTR types have been identified within a country and individual state, suggesting considerable variability of VNTR types in swine populations (Vranckx et al., 2011; Dos Santos et al., 2015; Takeuti

et al., 2017b). Among all types detected in different countries (i.e., United States, Mexico, Brazil, and Spain), a common *M. hyopneumoniae* VNTR type has not been detected (Dos Santos et al., 2015). In swine-dense geographical areas, multiple *M. hyopneumoniae* VNTR types have been identified in endemically infected herds and even in individual pigs (Vranckx et al., 2011; Nathues et al., 2011; Michiels et al., 2017; Tonni et al., 2021). Great homogeneity and/or identical *M. hyopneumoniae* VNTR types in herds that are in close geographical proximity, or herds that belong to the same production flow, has also been shown (Mayor et al., 2007; Charlebois et al., 2014; Pantoja et al., 2016; Takeuti et al., 2017b; Rebaque et al., 2018). In all cases, one distinct *M. hyopneumoniae* VNTR type along with multiple clonal types (i.e., similar VNTR types) have been described. It could be hypothesized that the degree of *M. hyopneumoniae* genetic variability among herds might be influenced by herd management. However, potential drivers and mechanisms of genetic heterogeneity have been poorly explored and defined.

1.3.5.2 Molecular characterization techniques

Several techniques have been standardized to molecularly characterize *M. hyopneumoniae* using nucleic acid amplification, including Amplified Fragment Length Polymorphism (AFLP; Kokotovic et al., 1999; Stakenborg et al., 2006), Restriction Amplified Polymorphic DNA analysis (RAPD; Artiushin and Minion, 1996; Vicca et al., 2003), Pulse-Field Gel Electrophoresis (PFGE; Stakenborg et al., 2006), Random Fragment Length Polymorphism (RFLP; Stakenborg et al., 2006), and DNA Microarrays (Madsen et al., 2007). However, variations in reproducibility, feasibility, and discriminatory power exist among these molecular techniques (Stakenborg et al., 2006;

Sibila et al., 2009). To provide further discrimination, Multiple-Locus Sequence Typing (MLST; Mayor et al., 2008), MLVA (Vranckx et al., 2011; Nathues et al., 2011; Dos Santos et al., 2015; Tamiozzo et al., 2015), complete or partial gene sequencing (Tsungda and Minion, 1998; Mayor et al., 2007; Garza-Moreno et al., 2019), and whole genome sequencing (Minion et al., 2004; Vasconcelos et al., 2005; Liu et al., 2011; Siqueira et al., 2013; Han et al., 2017) have been recently employed. While each molecular method has its strengths and disadvantages, it is important to consider the question at hand, resources available, and capabilities that each method can provide before utilizing such techniques.

Thus far, molecular characterization techniques for *M. hyopneumoniae* have been commonly employed in research settings. However, the application of *M. hyopneumoniae* characterization in field investigations has increased in the United States to provide veterinarians additional insight into the transmission and control of this microorganism. Although whole genome sequencing has been proposed as a highly descriptive and thorough method, the routine application of this technique for disease investigations may pose difficulties based on its current practicality, feasibility, and long turn-around time, as it applies to disease investigations. Moreover, pathogen isolates are commonly used for whole genome sequencing to achieve a high sequence coverage by limiting the presence of host DNA contamination that exists in clinical samples. However, one bottleneck for the whole genome sequencing of *M. hyopneumoniae* is the poor feasibility to obtain isolates. Therefore, the utilization of typing methods (i.e., MLVA) and complete or partial-gene sequencing for *M. hyopneumoniae* characterization have increased in veterinary diagnostic laboratories, as the control and elimination of this microorganism is widely attempted worldwide (Maes et al., 2018).

1.3.5.3 Typing methods

The most employed genomic typing methods for *M. hyopneumoniae* are MLST and MLVA. The MLST assay has been utilized for many bacterial species and was standardized for the molecular characterization of *M. hyopneumoniae* by Mayor et al. (2008). Several loci have been targeted, including putative and house-keeping genes (i.e., efp, metG, pgiB, recA, adk, rpoB, tpiA, gyrB, and gmk). As identified in Mycoplasma spp., house-keeping genes have been described to be highly conserved, resulting in limited variation among strains (Dumke et al., 2003). The MLVA assay is a typing method that identifies the number of VNTRs within surface proteins and has been employed for the molecular characterization of *M. hyopneumoniae* in Spain, Argentina, Belgium, United States, Mexico, and Brazil (de Castro et al., 2006; Vranckx et al., 2011; Dos Santos et al., 2015; Tamiozzo et al., 2015; Takeuti et al., 2017b; Michiels et al., 2017). Several techniques have described the *M. hyopneumoniae* variability using different loci numbers (e.g., 2 or 4) and types (i.e., P97, P146, H1, H4, H5). The targeted loci have been selected for their presumed bacterial adherent capability to the ciliated epithelium, presence of repetitive, tandem repeats, or a high degree of variability (Vranckx et al., 2011; Dos Santos et al., 2015; Tamiozzo et al., 2015; Rebaque et al., 2018). Currently, MLVA assay is commonly performed and has been implemented for epidemiological investigations due to its high discriminatory power, reproducibility, and use of clinical samples (Vranckx et al., 2011; Dos Santos et al., 2015). Despite this, discrepancies regarding the genomic characterization of *M. hyopneumoniae* and interpretation currently exist. Like other typing methods, MLVA evaluates few and

specific genomic areas. Therefore, other areas within the genome that may contribute to variability may be unrepresented.

1.3.5.4 Sequencing methods

Genomic sequencing methods have been developed to evaluate the entire genome or individual genes of *M. hyopneumoniae*. To further explore how the genomic structure of *M. hyopneumoniae* can influence pathogenesis and host-pathogen interaction, whole-genome sequencing has been attempted (Minion et al., 2004; Vasconcelos et al., 2005; Liu et al., 2011; Siqueira et al., 2013; Han et al., 2017). While this molecular technique offers descriptive and thorough information, the feasibility to routinely perform whole-genome sequencing may be limited due to cost, the difficulty of interpretation, computational requirements, and the need for sophisticated equipment. Therefore, complete or partial sequencing of individual *M. hyopneumoniae* genes has been attempted (Mayor et al., 2007; Felde et al., 2018). Due to the paralogous nature of P146 with P97, and the presence of distinct motifs and consensus regions (i.e., PQ, S, PS for locus P146), partial sequencing of the encoded gene mhp 684 (3954 bp) has been previously described (Mayor et al., 2007; Bogema et al., 2012). Throughout the employment of sequencing methods, the number of VNTRs has also been evaluated for *M. hyopneumoniae* (Felde et al., 2018; Garza-Moreno et al., 2019). However, as with MLVA, there is limited knowledge on the interpretation of sequencing methods and the insight gained from the comparison of molecular assays that evaluate nucleotide differences at the consensus or individual gene level.

1.3.5.5 Field application

Utilization of molecular techniques can aid in the characterization and genetic comparison of *M. hyopneumoniae* variants. In doing so, insight related to pathogen origin, transmission, and potential evolution can be gained. Nevertheless, molecular characterization techniques have not been commonly used in the field. One of the reasons is because genomic classification criteria for *M. hyopneumoniae* have not been established due to the limited comparison of currently available molecular techniques. Another reason is the poor understanding of the biological importance of genetic variability in *M. hyopneumoniae*. An improved understanding of the *M. hyopneumoniae* diversity via the comparison of commonly used molecular characterization techniques is warranted to aid in epidemiological investigations and pathogen tracing.

1.3.6 Surveillance

Surveillance is often defined as the 'ongoing and systematic collection, analysis, and interpretation of health data aimed at early detection of a specific disease or agent in a population, or identifying an elevated risk of acquiring an infectious disease to maximize prevention and control' (Thurmond, 2003). The design of surveillance strategies is a function of temporality and its ability to accurately identify the presence of an infectious agent in a population at any given time. Surveillance strategies should maximize the probability of true early detection while minimizing the probability of false-positive detection (Thurmond, 2003). Attributes that define a surveillance strategy include accuracy, precision, rapidity, efficiency, and value. Given their perceived risk for disease introduction to breeding herds, replacement gilts are routinely surveilled for significant pathogens, including *M. hyopneumoniae* (Neumann and Hall, 2019).

Historically, the design of surveillance strategies in replacement gilt populations has been based on the assumption of perfect accuracy (Cannon and Roe, 1982) and the capability for multi-pathogen testing. Nevertheless, the question for potential false diagnostic assurance arises from the poor diagnostic sensitivity described for *M. hyopneumoniae*, given certain sample types and timing (Pieters et al., 2017; Sponheim et al., 2020). Therefore, the ability to detect a recent *M. hyopneumoniae* infection using currently available sample types warrants further investigation to improve strategies to aid in disease prevention and control. Also, surveillance strategies were designed based on the fact that more recent sample techniques and diagnostic tools were not available or had not been researched. With the more recent development of alternative clinical samples and diagnostic tests, there is a need to re-evaluate current surveillance strategies

1.3.7 Control and elimination

1.3.7.1 Classification of herd health status

Initiation of a disease control plan centers around the assessment of the herd health status. Assessment of herd health status involves a comprehensive analysis of clinical and diagnostic data for both the recipient sow herds and incoming gilt populations, which can be utilized to best guide control strategies for the herd of interest. A classification of *M. hyopneumoniae* health status has been initially proposed by Garza-Moreno et al. (2018) and later by the American Association of Swine Veterinarians (AASV, 2019), which groups recipient sow herds and/or incoming replacement gilt populations into different categories based on the presence of clinical signs and lung lesions, and the detection of antibodies and/or genetic material for *M. hyopneumoniae*.

1.3.7.2 Management methods

Optimization of management practices and housing conditions to either lessen the spread of *M. hyopneumoniae* or reduce disease severity should be initially accomplished to control disease. Management of pigs using all-in, all-out production has been shown to control *M. hyopneumoniae* by allowing for breaks in pathogen transmission between populations of differing ages and health dynamics, tailored environmental conditions, and facility disinfection (Clark et al., 1991). Early weaning and parity segregation have also been employed to reduce pathogen transmission from dams to offspring (Alexander et al., 1980; Dee, 1994). However, the routine implementation of early weaning and parity segregation is not always feasible and can pose challenges with production. Reduction of herd size and animal stocking density and improvement of biosecurity measures can also be implemented to reduce the spread of *M. hyopneumoniae* within or between pig cohorts (Maes et al., 2008).

1.3.7.3 Gilt acclimation

Continued pathogen transmission and herd instability may exist in infected populations that receive naïve replacement gilts. In the swine industry, this challenge has become a norm due to economic, production, and health factors associated with purchasing high health replacement gilts (Pieters and Fano, 2016). Several strategies, including vaccination, gilt flow management, and controlled exposure, have been practiced to acclimate gilts to pathogens that are endemically present in the recipient herd, with the purpose of ensuring immunity and decreasing pathogen circulation (Fano and Payne, 2015; Garza-Moreno et al., 2018). Vaccination is the most common acclimatization method, and works by inducing immunity against *M. hyopneumoniae*

before entry into sow herds. However, this intervention's inherent limitations, such as incomplete protection from infection, has driven the utilization of other acclimation options. Since *M. hyopneumoniae* infection is usually maintained in herds, clinically affected older gilts and sows have been used as "seeders" to amplify and shed the bacterium to younger gilts. Nevertheless, the nature of the pathogen is the limiting factor for this type of exposure. Roos et al. (2016) proposed that a 1:1 ratio of seeder to susceptible gilts is necessary to ensure that all gilts have been exposed to the pathogen in a relatively short period of time (4 weeks). In the field, this method is not feasible and/or practical due to limited space and facility requirements and the large number of infected non-select or culled animals that would be to act as seeders. For the reason described above, controlled exposure of susceptible gilts to a herd-specific *M. hyopneumoniae* lung homogenate has been proposed and applied in the field (Robbins et al., 2016; Figueras et al., 2020). The control exposure strategy has also been utilized in *M. hyopneumoniae* elimination efforts to establish a homogeneous health status at the start of the closure (i.e., Day 0; Holst et al., 2015; Yeske et al., 2020).

1.3.7.4 Vaccines

Commercial vaccines, consisting of inactivated and adjuvanted whole-cell preparations, are commonly utilized to control *M. hyopneumoniae* infection. Studies have shown beneficial vaccination effects, including the reduction of *M. hyopneumoniae* bacterial load, improvement in growth performance, and decrease in disease severity (Maes et al.,1998; Maes et al., 1999; Fano et al., 2006b; Meyns et al., 2006; Sibila et al., 2007a; Vranckx et al., 2012a). Despite their wide use, partial protection against *M. hyopneumoniae* infection is induced (Meyns et al., 2006; Pieters et al., 2010; Villarreal et

al., 2011b). While the mechanisms for protection are not yet fully understood, the induction of localized mucosal antibodies and cell-mediated immunity has been suggested to play an important role in protection (Maes et al., 2020a). Different vaccination strategies have been adopted to optimize disease control, depending on the production stage, management practices, and herd infection dynamics. In the field, *M. hyopneumoniae* bacterins are often administered to weaned piglets since colonization has been shown to occur from the first weeks of life (reviewed by Haesebrouck et al., 2004; Sibila et al., 2007b) and to provide ample time for development of an immune response prior to disease presentation. Moreover, incoming replacement gilts are often vaccinated for acclimation purposes (Garza-Moreno et al., 2018).

Vaccine efficacy has been shown to vary based on vaccination timing and the number of doses and is improved when multiple doses are administered (Baccaro et al., 2006; Sibila et al., 2007a, Arsenakis et al., 2017; Garza-Moreno et al., 2019). However, the indirect effect of vaccination on reducing infection pressure in a population remains poorly understood. Several studies described a numerical reduction in *M. hyopneumoniae* transmission when one or two vaccine doses were administered to either infected or susceptible pigs (Meyns et al., 2006; Pieters et al., 2010; Villarreal et al., 2011). Nevertheless, the combined effect of multiple vaccinations in both populations, infected and susceptible gilts, on *M. hyopneumoniae* transmission remains unclear. Given that susceptible subpopulations are often present in an infected herd, additional research to assess the vaccinations' role in *M. hyopneumoniae* infection dynamics is warranted.

1.3.7.5 Antibiotics

Antibiotics are considered paramount for the control of *M. hyopneumoniae* infection. Antibiotics effective against *M. hyopneumoniae* include tetracyclines, 15-and 16-membered lactone ring macrolides, fluoroquinolones, lincosamides, florfenicol, and aminoglycosides (Gautier-Bouchardon, 2019). *Mycoplasma hyopneumoniae* is intrinsically resistant to antibiotics that target cell wall synthesis (e.g., β-lactams), as well as to 14-membered lactone ring macrolides. Studies have shown antibiotics to reduce the bacterial load and to mitigate *M. hyopneumoniae*-associated disease (Del Pozo Sacristan, 2014; Maes et al., 2017). In addition, antibiotics are commonly employed in *M. hyopneumoniae* elimination strategies (Holst et al., 2015). Despite their known antibacterial effects, there has been no information suggesting that antibiotics alter the transmissibility of *M. hyopneumoniae.* Knowledge on the effect of antibiotics on transmission would guide the time at which antibiotic treatment is applied to lessen pathogen spread, especially for outbreak scenarios. Post-treatment, the persistence of *M. hyopneumoniae* infection has been suspected due to the detection of genetic material by real-time PCR in clinical samples collected from treated pigs (Thacker et al., 2006; Le Carrou et al., 2006; Painter et al., 2012), or the lingering of clinical signs. Acquired antibiotic resistance has been described for *M. hyopneumoniae* (Maes et al., 2020b), but it has been evaluated to a limited extent due to bacterial isolation challenges. Therefore, the infectious potential of the bacterium post-antibiotic treatment has not been investigated *in-vivo.*

1.3.7.6 Elimination strategies

Overall, the foundation of a successful elimination program is built mainly upon the ability to prevent pathogen transmission, reduce infection prevalence, and later

confirm freedom of disease. While this concept can pertain to almost any pathogen, elimination protocols are often tailored towards a specific infectious agent. For *M. hyopneumoniae*, four elimination strategies, namely 1) depopulation and repopulation; 2) partial depopulation (Swiss Method); 3) Herd closure and medication; 4) Whole herd medication, have been mostly employed (Holst et al., 2015). The process for strategy selection is often dependent upon several factors, including but not limited to herd structure, the ability for continued production, feasibility, available resources, and the likelihood of success.

Table 1.1 Classification and clinical-pathological presentation of common swine Mycoplasmas

Mycoplasma species	Taxonomic classification (group; subgroup)	Generalized pathogen-host interaction	Clinical-pathological presentation	References
M. hyopneumoniae	Hominis; Neurolyticum	Pathogenic	Pneumonia	Goodwin et al., 1965; Maré and Switzer, 1965
M. hyorhinis	Hominis; Neurolyticum	Opportunistic	Polyserositis Arthritis Otitis Pneumonia Meningoencephalitis Conjunctivitis	Friis, 1975; Friis and Feenstra, 1994; Roberts et al., 1963; Morita et al., 1998; Lin et al., 2006; Pereira et al., 2017; Luehrs et al., 2017; Resende et al., 2019
M. hyosynoviae	Hominis; Hominis	Opportunistic	Arthritis	Ross and Duncan, 1970; Hagedorn-Olson et al., 1999a
M. suis (formerly known as *Eperythrozoon suis*)	Haemominutum	Pathogenic	Anemia	Splitter, 1950
M. flocculare	Hominis; Neurolyticum	Commensal		Meyling and Friis, 1972
M. hyopharyngis	Hominis; Lipophilum	Commensal		Erickson et al., 1986

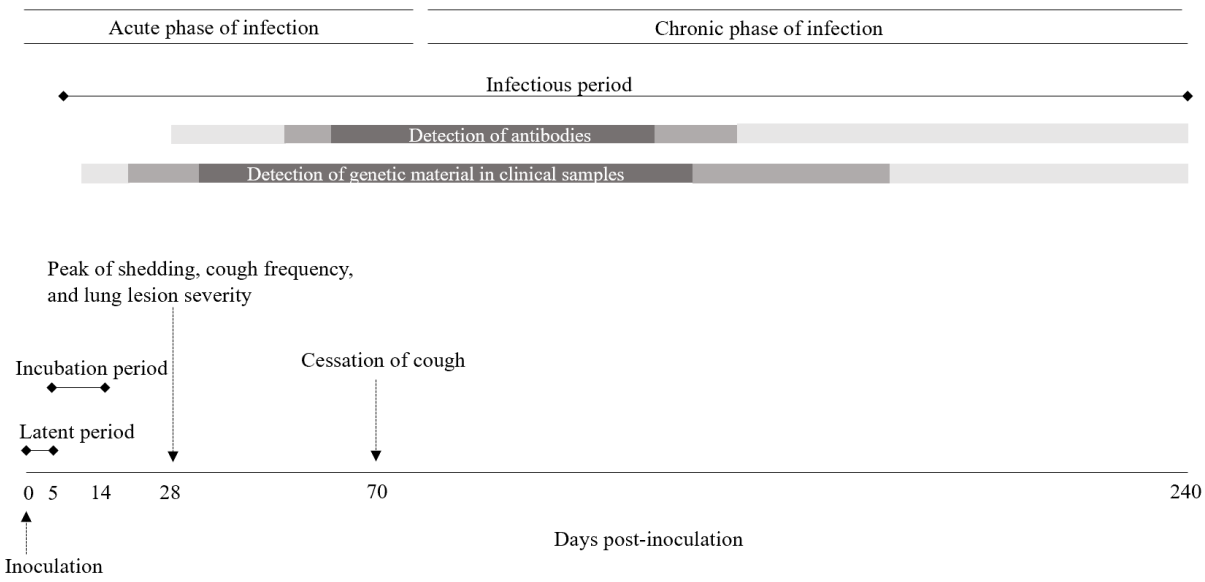

Figure 1.1 Timeline of infection dynamics and detection of *Mycoplasma hyopneumoniae* under experimental conditions

Gray shading represents the likelihood of genetic material or antibody detection for *M. hyopneumoniae*, with darkest gray shade having the highest likelihood. Onset and duration of gray shading is proposed based on summarized information of *M. hyopneumoniae* detection described in the literature.

Chapter 2: Natural transmission of *Mycoplasma hyopneumoniae* in a naïve gilt population

Work from this chapter has been published in:

Betlach, A.M., Valeris-Chacin, R., Singer, R.S., Allerson, M., Pieters, M. 2020. Natural transmission and detection of *Mycoplasma hyopneumoniae* in a naïve gilt population. **Veterinary Microbiology.** 248: 108819.

2.1 Summary

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) continues to be a prevalent and economically important swine respiratory pathogen. For *M. hyopneumoniae* surveillance, blood samples and/or oral fluids are commonly collected from incoming replacement gilts prior to entering sow herds. However, limitations to this approach exist, particularly due to low sensitivity during acute stages of natural infection, leading to diagnostic uncertainty. Therefore, the objective of this study was to evaluate the natural transmission and detection of *M. hyopneumoniae* based on the introduction of one infected gilt to a naïve population. Twenty-nine naïve gilts were housed with one *M. hyopneumoniae* naturally exposed gilt for 8 weeks. Deep tracheal catheters, laryngeal swabs, and blood samples were individually collected from each gilt at 0, 1, 2, 4, 6, and 8 weeks post-contact (wpc), along with one pen-based oral fluid sample. Blood samples were assayed by ELISA, while all other samples were tested by real-time PCR. The transmission rate of *M. hyopneumoniae* (β) was estimated using a Bayesian mixed-effects generalized linear model. At 8 wpc, 27% (8/29) of the naïve gilts had become infected (β=0.73 new infected gilts/gilt-week). Seroconversion was detected in 3% of contact gilts at 8 wpc. Oral fluids were negative for *M. hyopneumoniae* at all samplings. In this study, the natural transmission of *M. hyopneumoniae* was slow and detection varied based on sample type and timing. Thus, *M. hyopneumoniae* surveillance protocols should include lower respiratory tract samples that are tested by real-time PCR to avoid the introduction of potentially infected gilts into naïve sow herds.

2.2 Introduction

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is a host-specific bacterium that causes enzootic pneumonia in swine (Maré and Switzer, 1965; Goodwin et al., 1965). Signs suggestive of *M. hyopneumoniae* infection consist of dry cough, labored breathing, and reduced growth rate. *Mycoplasma hyopneumoniae* is mainly transmitted through direct contact between infected and susceptible pigs (Pieters and Maes et al., 2019).

Replacement gilt introduction is practiced in the swine industry for genetic advancement, maintenance of parity distribution, and productivity of sow herds (Patterson and Foxcroft, 2019). Nevertheless, replacement gilts are perceived as a risk for pathogen entry due to their frequent introduction year-round. Biosecurity practices, including varying isolation periods (1-6 weeks), and/or vaccination, and routine surveillance testing prior to sow herd entry are implemented to minimize pathogen introduction and to assure gilt health status (Neumann and Hall, 2019). The most common surveillance protocol entails testing 30 individual gilt blood samples by ELISA, assuming perfect accuracy (Cannon and Roe, 1982), along with PCR testing of aggregate samples (e.g. oral fluids; Rotolo et al., 2017). Blood samples and oral fluids are the most utilized sample types due to ease of collection and ability for multi-pathogen testing. However, the ability to detect multiple pathogens in one sample can vary significantly given differences in the pathogenesis, diagnosis, and epidemiology between pathogens.

The detection of *M. hyopneumoniae* or its antibodies in oral fluids or serum, respectively, is challenging in the acute stages of infection (Pieters et al., 2017) due to poor diagnostic sensitivity and delayed seroconversion, thus leading to false diagnostic

assurance. Gilts are often vaccinated against *M. hyopneumoniae* prior to entering the sow herd, which could further complicate the interpretation of serological results. Moreover, under experimental conditions, the spread of *M. hyopneumoniae* in a population has been described to be slow, ranging from 0.56-1.16 new infections per infected pig in a 6-week period (Meyns et al., 2004, Villarreal et al., 2011). Nevertheless, *M. hyopneumoniae* transmission and detection may vary in naturally infected pigs. Therefore, the objective of this study was to evaluate the natural transmission and detection of *M. hyopneumoniae* based on the introduction of one infected gilt to a naïve population.

2.3 Materials and methods

2.3.1 Animals and housing

Thirty-two 7-week old gilts were obtained from a *M. hyopneumoniae* and Porcine Reproductive and Respiratory Syndrome Virus negative source and were housed at a filtered biosafety level-2 experimental isolation unit. A history of more than five years of negativity to *M. hyopneumoniae* infection, absence of clinical signs, and supportive diagnostic records were used to verify health status. *Mycoplasma hyopneumoniae* naive status was confirmed in all gilts via the absence of antibodies and lack of detection of *M. hyopneumoniae* using deep tracheal catheters tested by real-time PCR. The study was conducted according to project-specific protocols, which were approved by the Swine Services Unlimited Inc. ethical review board, based on institutional animal care and use guidelines. The approval number for this study was PSI-1-2018-11Dec18.

3.3.2 Study design

A graphical representation of the study design is provided in Figure 2.1. Briefly, gilts were transported to the isolation unit at two different time points for the different study phases, namely 1) Development of natural seeder and 2) Natural transmission. Upon arrival, gilts were randomly selected and ear-tagged. For the first study phase, two gilts were experimentally inoculated with *M. hyopneumoniae* and housed with one contact gilt until natural infection in the contact gilt (natural seeder) occurred. For the second study phase, the natural seeder was relocated to a clean, experimental room, where it was housed with 29 contact naïve gilts for eight weeks. As a result, the initial *M. hyopneumoniae* prevalence was 3%. To assess transmission and detection, blood, laryngeal swabs, and deep tracheal catheters were individually collected from each gilt at 0, 1, 2, 4, 6, and 8 weeks post-contact (wpc), along with the collection of one pen-based oral fluid sample. Gilts were sampled at these specific time points to mimic various isolation lengths that are implemented in the field. At the end of the study, gilts were humanely euthanized using electrocution and bronchial swabs and lung tissue samples were collected, along with the evaluation of lung lesions.

2.3.2.1 Development of natural seeder

Two gilts were experimentally inoculated with 15 mL of lung homogenate containing $1x10^5$ CCU/mL of a moderately virulent *M. hyopneumoniae* strain (232; Minion et al., 2004) via intra-tracheal (Gomes Neto et al., 2014). The experimentally infected gilts were confirmed *M. hyopneumoniae* positive by real-time PCR in laryngeal swabs and deep tracheal catheters at 7 days post-inoculation (dpi) and were comingled in the same pen (1.02 m^2/gilt) with a contact naïve gilt to induce the development of a

natural seeder. Laryngeal swabs and deep tracheal catheters were collected from the contact gilt every 3 to 6 days to monitor for infection. Once the contact gilt was confirmed *M. hyopneumoniae* positive in both sample types, the gilt was considered a natural seeder, assigned a new ear tag and was relocated to a different room for the second study phase. The experimentally infected gilts were euthanized at this time point.

2.3.2.2 Natural transmission

At day 0, the natural seeder was commingled with 29 contact gilts in a separate experimental room. Gilts were housed in single pen (1.45 m^2/gilt) throughout the duration of the study. At 0, 1, 2, 4, 6, and 8 wpc, blood, laryngeal swabs and deep tracheal catheters were collected from each individual gilt, along with one oral fluid sample at the pen level. Investigators were masked to gilt identification to avoid sampling bias. At each sampling event, samples were collected in a routine fashion in this order: laryngeal swab, deep tracheal catheter and blood. The sampling equipment was disinfected between individual gilts to avoid cross-contamination. In addition, individual coughs were recorded daily for 30 min at the same time of the day. All gilts were humanely euthanized at 8 wpc, followed by collection of bronchial swabs and lung tissue samples, and evaluation of lung lesions.

2.3.3 Sample collection and processing

Laryngeal swabs were collected from each gilt as previously described by Pieters et al. (2017), using sterile collection swabs (BBL™ CultureSwab™, Sparks, MD, USA), mouth speculum, and using a hemostat to lengthen the swab reach as gilts aged (Sponheim et al., 2020). Deep tracheal catheters were collected by intra-tracheally

introducing a sterile, red rubber catheter (Covidien, Mansfield, MA, USA) into the mouth of each gilt, as described by Fablet et al. (2010). After collection, the tip of each catheter was inserted into a sterile 5mL tube containing 500ul of phosphate-buffered saline and later vortexed to obtain tracheal secretions. Oral fluid samples were collected as previously described by Prickett and Zimmerman (2010). Briefly, a 60.9 cm long cotton rope was hung in the center of the pen and suspended at pig shoulder height. Oral fluids were manually extracted and transferred to sterile tubes. Bronchial swabs were collected from each gilt after euthanasia as described by Pieters et al. (2009). Laryngeal and bronchial swabs, deep tracheal catheters, and oral fluids were stored at -20^{o}C until processing, and were individually processed for DNA extraction using MagMAX^{TM}-96 Viral RNA isolation kit and MagMAX^{TM} Express-96 Magnetic Particle Processor (Life Technologies, Grand Island, NY, USA). The extracted genetic material was tested for *M. hyopneumoniae* using species-specific real-time PCR with VetMAX^{TM} qPCR Master Mix and VetMAX^{TM} *M. hyopneumoniae* Reagents kit (Life Technologies, Grand Island, NY, USA), following manufacturers protocol. Samples were considered positive with a <40 Ct value.

Blood samples were submitted to the University of Minnesota, Veterinary Diagnostic Laboratory (UMN-VDL) for the detection of *M. hyopneumoniae* antibodies using an indirect ELISA test (Idexx Laboratories, Westbrook, ME, USA). Samples were considered positive with a ≥ 0.4 S/P ratio.

Gross lung lesions were scored on a scale from 0 to 100% depending on lesion severity and anatomical lobe (Pointon et al., 1999). Lung tissue samples were collected in the transitional area when lesions were evident or in a consistent area if lesions were

absent. Tissue samples were fixed in 10% buffered formalin and evaluated for histopathology at the UMN-VDL. Microscopic lung lesions were assessed and scored on a scale from 0 to 4 as previously described (Thacker et al., 1999).

2.3.4 Diagnostic criterion

Gilts were determined to be infected with *M. hyopneumoniae* based on a parallel interpretation of diagnostic results at the individual level, thus if either of the real-time PCR results were positive, the corresponding gilt was considered infected.

2.3.5 Data analysis

Antemortem samples were compared by evaluating the time-to-detection for *M. hyopneumoniae* based on a Cox Proportional Hazard Regression model, while incorporating shared frailty for gilt. The proportional hazards assumption was assessed using *cox.zph* function from the package survival (Therneau, 2015) in R Studio® version 3.6.1 (R Core Team, 2019, Vienna, Austria). A p-value <0.05 was used to determine statistical significance. *Mycoplasma hyopneumoniae* transmission parameters were evaluated using a susceptible-infectious (SI) model. Equal susceptibility was assumed, and *M. hyopneumoniae* positive gilts remained infectious for the duration of the study with a lack of recovery. The transmission rate (β) per week was estimated during the 8-week period of the study (t=8) using a Bayesian mixed-effects generalized linear model with family binomial, complementary log-log link function and a log I/N (ratio of infected(I)/total population(N)) as offset, while incorporating gilt as random intercept. The model was conducted using Stata 16 (StataCorp, Release 16, 2019, College Station, TX, USA). The probability that a susceptible gilt became infected was $1\text{-}e^{-\beta \Delta t I/N}$ (Velthuis

et al., 2003). The transmission rates from previous *M. hyopneumoniae* transmission studies (Meyns et al., 2004; Villarreal et al., 2011; Roos et al., 2016) were used to build a prior distribution. As a result, a normal prior for the log transmission rate with a mean and variance of -0.12 and 1.59, respectively, was employed. Four Markov chain Monte Carlo chains were run with 100,000 iterations each after a burn-in of 5,000 iterations. The adjusted reproductive ratio (R_n) was estimated by multiplying β by the duration of infection. Since *M. hyopneumoniae* can shed for up to 214 days (Pieters et al., 2009), the duration of infection was equivalent to the length of study.

2.4 Results

2.4.1 Development of natural seeder

At 7 dpi, both experimentally inoculated gilts were *M. hyopneumoniae* positive by real-time PCR (Ct values 20.19 and 25.06). At 23 dpc, *M. hyopneumoniae* was detected by real-time PCR in both the deep tracheal catheter and laryngeal swab (Ct values 35.7 and 36.2, respectively) in the contact gilt, thus confirming the development of a natural seeder.

2.4.2 Natural transmission

2.4.2.1 Ante-mortem detection of M. hyopneumoniae*, seroconversion, and clinical signs*

Detection of *M. hyopneumoniae* by real-time PCR in all sample types and serological detection of antibodies in the natural seeder and contact naïve gilts is shown in Table 2.1. Throughout the natural transmission phase (0-8 wpc), *M. hyopneumoniae* was consistently detected by real-time PCR in deep tracheal catheters collected from the natural seeder, resulting in 100% sensitivity. Detection of *M. hyopneumoniae* by real-time PCR in laryngeal swabs showed 83.3% sensitivity, as one false negative result was

obtained at 4 wpc. The natural seeder was seropositive for *M. hyopneumoniae* at 6 and 8 wpc, resulting in 33.3% sensitivity in blood samples by ELISA.

At 0 wpc, all contact gilts (n=29) were confirmed *M. hyopneumoniae* negative in deep tracheal catheters and laryngeal swabs. Contact gilts remained negative to *M. hyopneumoniae* detection by real-time PCR until 4 wpc. At 6 wpc, 3.4% (1/29) of contact gilts tested positive for *M. hyopneumoniae* in laryngeal swabs and deep tracheal catheters. At 8 wpc, *M. hyopneumoniae* was detected in 3.4% (1/29) and 17.2% (5/29) of contact gilts in laryngeal swabs and deep tracheal catheters, respectively, and 3.4% (1/29) of contact gilts were seropositive.

When analyzing with the Cox Proportional Hazard Regression model, the hazard of detection of secondary *M. hyopneumoniae* infections in deep tracheal catheters by real-time PCR was 12.67 times the hazard of detection of secondary *M. hyopneumoniae* infections in blood samples by ELISA (95% CI: 1.3, 123.3; p=0.02). The hazard ratio of *M. hyopneumoniae* detection by real-time PCR in deep tracheal catheters compared to laryngeal swabs was 6.96 (95% CI: 0.79, 61.3; p=0.08). Moreover, the hazard ratio of detection of secondary *M. hyopneumoniae* infections in laryngeal swabs when using real-time PCR was 1.82 times the hazard of detection of secondary *M. hyopneumoniae* infections in blood samples when using ELISA (95% CI: 0.10, 31.9; p=0.68). Assumption of hazard proportionality was met, suggesting that the hazard ratio of *M. hyopneumoniae* detection, given any of the two evaluated sample types, was constant over time. The overall effect of sample type on time-to-detection of *M. hyopneumoniae* did not reach statistical significance (p=0.06). All oral fluids were *M. hyopneumoniae* negative by real-time PCR throughout the study. Coughing was observed in the natural

seeder and in one *M. hyopneumoniae* infected contact gilt at 3.3 wpc and 7.5 wpc, respectively. Coughing gilts were also *M. hyopneumoniae* positive by real-time PCR prior to the onset of coughing.

2.4.2.2 Postmortem detection of M. hyopneumoniae *and lung lesion scores*

At necropsy (8wpc), *M hyopneumoniae* was detected by real-time PCR in bronchial swabs collected from the natural seeder and 27.6% (8/29) of contact gilts. A high percentage (41%) of cranio-ventral consolidation and moderate (grade 2) microscopic lesions were observed in the lungs of the natural seeder. For the contact gilts, 55.2% (16/29) and 3.4% (1/29) showed either mild (≤10%) or moderate (11-20%) gross lung lesions suggestive of *M. hyopneumoniae* infection. Regarding microscopic scores, 31.0% (9/29), 24.1% (7/29), 17.2% (5/29), and 13.8% (4/29) of contact gilts showed mild (grade 1), moderate (grade 2), severe (grade 3), or very severe (grade 4) lung lesions, respectively.

2.4.2.3 Transmission assessment

Based on the diagnostic criterion, 27% (8/29) of contact gilts became *M. hyopneumoniae* positive during the 8-week period. The transmission rate per week (β) was 0.73 with a 95% highest posterior density credible interval of 0.31-1.61. At 6 and 8 wpc, the *M. hyopneumoniae* incidence rate was 0.006 and 0.035, respectively. The R_n value (95% confidence) was estimated as 5.84 (2.48-12.88), implying that a mean of 5.84 secondary infections resulted from one naturally infected gilt at 8 weeks post-introduction in the group of naïve gilts.

2.5 Discussion

This study was designed to evaluate the transmission and detection of *M. hyopneumoniae* based on the introduction of one naturally infected gilt to a naïve population. Our results showed that the estimated transmission rate (β) and eight-week adjusted reproductive ratio (R_n) of *M. hyopneumoniae* was 0.73 new infected gilts/gilt-week and 5.84 secondary infected gilts/8 weeks, respectively, with a secondary infection initially detected at 6 wpc to the natural seeder. The ability to detect a recent *M. hyopneumoniae* introduction was largely influenced by the sample type and the length of time pigs were comingled prior to sampling.

The spread of *M. hyopneumoniae* has been described to be slow and persistent in swine populations. Assessment of *M. hyopneumoniae* transmission rate and duration of shedding has been primarily investigated in experimental trials. Nevertheless, the transmission and detection of *M. hyopneumoniae* may differ under natural conditions, which is important to consider when making inferences to the field. Compared to previous investigations (Meyns et al., 2004, Villarreal et al., 2011, Roos et al., 2016), the β and the R_n estimated in this study differed numerically. Several factors, including but not limited to, the type of infection, strain and infectious dose utilized, time and proportion of seeders that are introduced, and contact period, can directly influence the transmission parameters. Numerical differences in transmission and infection dynamics have been shown to occur between strains of high and low virulence (Meyns et al., 2004; Villarreal et al., 2011). Moreover, a long contact period can theoretically increase β in a susceptible population as the likelihood of effective contacts is often high under such conditions. In this study, a moderately virulent *M. hyopneumoniae* strain (232) was utilized during the development of the natural seeder. Although infectivity of the natural

seeder was unknown, a low relative bacterial load was detected at the time of initial introduction. Regardless, the β and the R_n estimated in this study were higher when compared to other *M. hyopneumoniae* transmission studies (Meyns et al., 2004; Villarrreal et al., 2011). Such differences could have resulted from the longer contact period that was employed in this study (i.e. 8 wpc versus 6 weeks by Meyns et al. (2004) and Villarreal et al. (2011)), resulting in the ability to detect an exponential increase in *M. hyopneumoniae* prevalence which specifically occurred between 6 and 8 wpc. It is important to mention that a single replicate was conducted in this study, thus resulting in a large confidence interval for the estimated transmission parameters.

Exposure to *M. hyopneumoniae* and consequently the establishment of infection and disease development are often dependent upon several potential risk factors, including managerial, environmental, and husbandry-related (Stärk, 2000; Maes et al., 2008). While differences in farm practices exist, it is important to consider what risk factors could influence the contact of a susceptible individual or population to the pathogen. In this study, gilts were housed in a single pen located in a mechanically ventilated and filtered air space. Compared to the field, different conditions related to ventilation, herd size, stocking density, and housing, etc., are likely to be employed, which may alter the transmission rate of *M. hyopneumoniae.*

Due the slow nature of infection and tropism towards respiratory cilium, differences in diagnostic sensitivity for *M. hyopneumoniae* detection given the sample type and stage of infection have been well-described (Sorensen et al., 1997; Sibila et al., 2009; Pieters et al., 2017, Sponheim et al., 2020). In the present study, the sensitivity for ante-mortem detection of *M. hyopneumoniae* in the natural seeder was greatest in deep

tracheal catheters, followed by laryngeal swabs and then blood samples. Regarding the contact gilts, a difference in the time-to-detection between deep tracheal catheters and blood samples was observed. Moreover, poor sensitivity for *M. hyopneumoniae* detection at the pen-level using oral fluids was apparent, as all samples were negative regardless of the presence of at least one infected gilt in the population. These findings are in agreement with previous studies that concluded *M. hyopneumoniae* detection to be dependent on sample type (Pieters et al., 2017, Sponheim et al., 2020). Veterinarians and producers rely heavily on diagnostic results to guide health and management decisions. Based on the conditions of this study, false negative results were more likely to occur in blood samples and oral fluids compared to deep tracheal catheters and laryngeal swabs. Therefore, these results suggest that there is a high likelihood of missing a recent *M. hyopneumoniae* introduction when considering the common surveillance protocols that are currently employed in the swine industry. Further research is needed to provide insight on detection probabilities given certain sampling strategies and isolation lengths for the surveillance of *M. hyopneumoniae.*

2.6 Conclusions

In this study, secondary *M. hyopneumoniae* infections post-introduction of a naturally exposed gilt were initially detected at 6 wpc. Moreover, the ability to detect *M. hyopneumoniae* was greatly influenced by the sample type and the length of contact, with blood samples and oral fluids leading to high likelihood of missed infections for eight weeks. Information generated in this study can be utilized by veterinarians and producers to modify current surveillance protocols with different *ante-mortem* sample types and/or

sample timing to increase sensitivity for *M. hyopneumoniae* detection, thus aiding in the prevention of *M. hyopneumoniae* outbreaks.

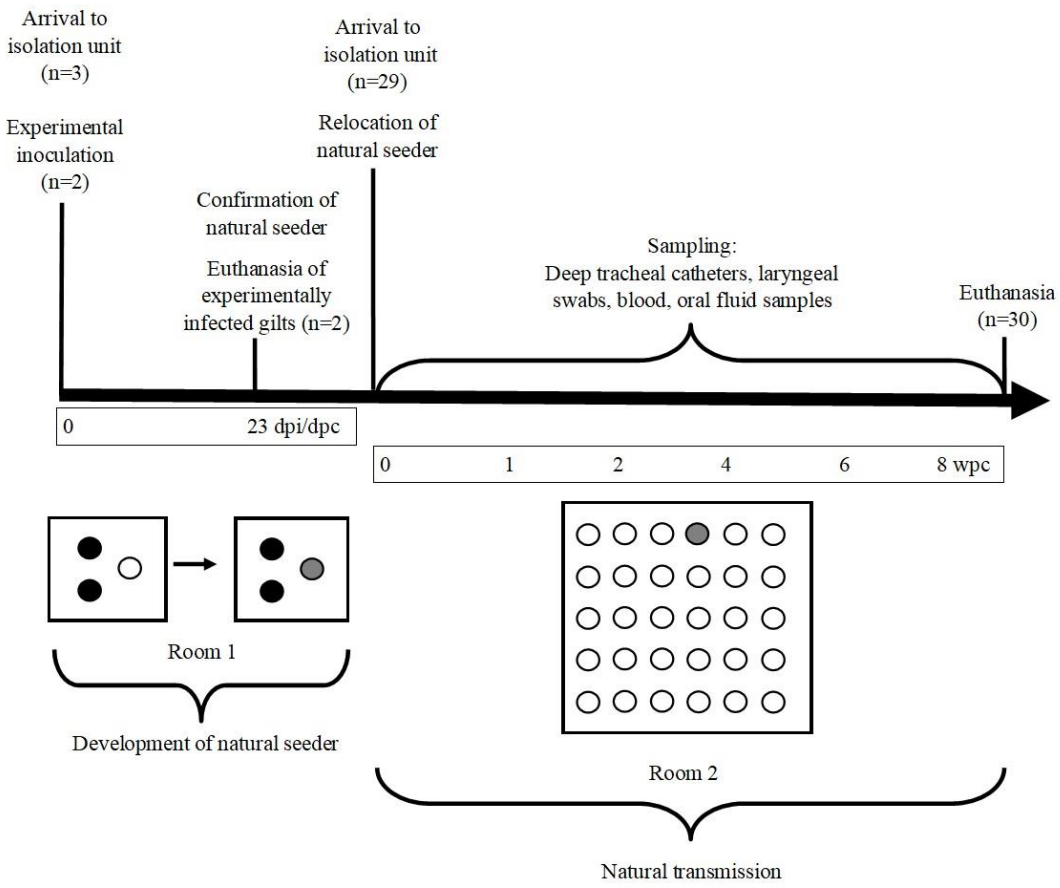

Figure 2.1 Graphical representation of study design and timeline

●=Experimentally infected gilt; ◉=Natural seeder; ○=Naïve contact gilt.

dpi=days post-inoculation; dpc=days post-contact; wpc=weeks post-contact.

Table 2.1 *Mycoplasma hyopneumoniae* detection and seroconversion during the natural transmission phase (0 to 8 weeks post-contact)

wpc	0		1		2		4		6		8		
Sample	**DTC**	**LS**	**DTC**	**LS**	**DTC**	**LS**	**DTC**	**LS**	**DTC**	**LS**	**DTC**	**LS**	**BS**
Natural seeder	**36.24**	**35.71**	**30.07**	**39.71**	**30.09**	**30.96**	**34.38**	**-**	**26.04**	**29.70**	**29.81**	**32.19**	**25.74**
Contact gilt(s) 1	-	-	-	-	-	-	-	-	-	-	-	-	**26.30**
2	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	**28.61**	**33.36**	**23.89**	**34.00**	**22.62**
4	-	-	-	-	-	-	-	-	-	-	**29.50**	-	**23.53**
5	-	-	-	-	-	-	-	-	-	-	**38.60**	-	**39.51**
6	-	-	-	-	-	-	-	-	-	-	**34.45**	-	**35.37**
7	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-	-	-	-	**38.07**
16	-	-	-	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	-	-	-	-	-

26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
27	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**37.47**
28	-	-	-	-	-	-	-	-	-	-	**38.92**	-	**38.69**		
29	-	-	-	-	-	-	-	-	-	-	-	-	-		
% contact gilts positive	0% (0/29)		0% (0/29)		0% (0/29)		0% (0/29)		**3.4%** (1/29)		**27.6%** (8/29)				
Oral fluid (pen)	(-)		(-)		(-)		(-)		(-)		(-)				

wpc=weeks post-contact. DTC=deep tracheal catheter sample. LS=laryngeal swab. BS=bronchial swab. Real time-PCR results are presented as negative (-) or positive (Ct value <40) for *M. hyopneumoniae*. Percent of positive contact gilts at each sampling event (0-8wpc) were based on parallel diagnostic interpretation of results. Time (wpc) at which gilt(s) were seropositive for *M. hyopneumoniae* is highlighted bold square boxes

Chapter 3: Effect of multiple vaccinations on transmission and degree of *Mycoplasma hyopneumoniae* infection in gilts

Work from this chapter has been published in:

Betlach, A.M., Fano, E., VanderWaal, K., Pieters, M. 2021. Effect of multiple vaccinations on transmission and degree of *Mycoplasma hyopneumoniae* infection in gilts. **Vaccine**. 39(4): 767-774.

3.1 Summary

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) infections continue to result in significant respiratory challenges in the swine industry worldwide. Vaccination for *M. hyopneumoniae* is commonly utilized, as reduction in bacterial loads and clinical severity in vaccinated pigs have been shown. However, the effect of *M. hyopneumoniae* vaccination on transmission across different populations has been minimally investigated. The aim of this pilot study was to evaluate the effect of multiple vaccinations on *M. hyopneumoniae* infection, transmission, and genetic variability in infected and susceptible gilt populations. Thirty-two naïve gilts were allocated to four treatment groups: 1) Vaccinated seeder (VS); 2) Non-vaccinated seeder (NVS); 3) Vaccinated contact (VC); and 4) Non-vaccinated contact (NVC). At 5, 7, and 9 weeks of age, all gilts selected to be vaccinated received a commercial *M. hyopneumoniae* bacterin for a total of 3 doses. At 11 weeks of age, VS and NVS gilts were inoculated with *M. hyopneumoniae* to become seeders. At 28 days post-inoculation (dpi), VS and NVS gilts were individually relocated to clean experimental rooms, where they were placed in contact with one age-matched VC or NVC gilt (1:1 ratio) for 14 days. Blood samples, tracheal samples, bronchial swabs, and lung lesions were collected and/or evaluated for *M. hyopneumoniae* infection. In this study, a three-dose vaccination strategy against *M. hyopneumoniae* significantly reduced bacterial load in seeder gilts. Furthermore, a numerical reduction in *M. hyopneumoniae* lung lesions at 28 dpi was observed in VS gilts. All VC gilts in the VS:VC treatment group pairing remained *Mycoplasma hyopneumoniae* negative, compared to other groups in which 1-2 transmission events occurred per treatment group. Results from this investigation provide insight in the

potential impact of multiple vaccinations on reducing *M. hyopneumoniae* transmission and infection. Further research encompassing vaccination of gilt groups in field settings is necessary to validate findings.

3.2 Introduction

Infections caused by *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*), the etiologic agent of enzootic pneumonia in swine (Maré and Switzer, 1965; Goodwin et al., 1965) continue to inflict significant respiratory health challenges in pigs worldwide. Clinical manifestations of *M. hyopneumoniae* infection are usually evidenced in the grower-finisher phase of production. However, infection at the sow herd is known to be influential on disease outcome in market pigs (Fano et al., 2007; Sibila et al., 2007a). Transmission of *M. hyopneumoniae* primarily occurs horizontally through nose-to-nose contact between infected and susceptible pigs (Morris et al., 1995) and through dam-to-piglet contact during the lactation period (Calsamiglia et al., 2000a; Pieters et al., 2014). Indirect transmission via aerosol has been considered to play an important role in the spread of *M. hyopneumoniae,* as studies have shown the likelihood of disease introduction to decrease as distance from positive herds increased (Goodwin, 1985; Jorsal and Thomsen, 1988). In addition, *M. hyopneumoniae* detection and viability in air samples collected up to 9.2 km from an infected herd has been described (Otake et al., 2010). Previous studies have also shown a reduction in the risk of *M. hyopneumoniae* transmission via entry of personnel and fomites by implementing biosecurity measures between infected and naïve herds (Batista et al., 2004; Pitkin et al., 2011).

To mitigate *M. hyopneumoniae* infection, parity segregation, all-in/all-out flow management, antibiotic treatments, vaccinations, among others have been implemented in

the field (Maes et al., 2007). Vaccinations against *M. hyopneumoniae* are commonly utilized in the field to decrease clinical severity of infection and associated disease (Sibila et al., 2007a; Haesebrouck et al., 2004). In commercial herds, *M. hyopneumoniae* vaccines are often administered to piglets prior to/during weaning, or to incoming replacement gilts (Haesebrouck et al., 2004; Garza-Moreno et al., 2018). The routine vaccination of sows against *M. hyopneumoniae* is minimally practiced (Garza-Moreno et al., 2018). However, sow vaccination is implemented to aid in herd stability or disease elimination (Bargen et al., 2004; Holst et al., 2015). Although complete protection is not achieved (Meyns et al., 2006; Pieters et al., 2010; Villarreal et al., 2011) , vaccination efficacy has been demonstrated by an improvement in average daily gain (2-8%) and feed efficiency (2-5%), reduction in bacterial load within the respiratory tract, decrease in clinical signs and lung lesions, and lower *M. hyopneumoniae* herd prevalence, especially when two doses are administered (Sibila et al., 2007a; Maes et al., 1998; Maes et al., 1999; Baccaro et al., 2006). Such beneficial outcomes may lead to a reduction in transmission among infected and susceptible pigs and influence the overall infection pressure at herd level.

Intra-herd dynamics of *M. hyopneumoniae* infection in sow herds are greatly influenced by the health status of gilts and the recipient sows. In endemically infected herds, the maintenance of infection has been suggested to occur from the consistent influx of negative replacement gilts, thus creating the opportunity for the pathogen to be transmitted between infected recipient sows and negative gilts, further propagating infection within the herd (Dee, 1996; Takeuti et al., 2017b; Pieters and Maes et al. 2019). Therefore, gilt acclimation strategies have been utilized to promote herd stabilization and

cessation of bacterial shedding during lactation. Several vaccination strategies with varied vaccine timing and number of doses have been applied in gilt acclimation programs (Garza-Moreno et al., 2018). However, the efficacy and potential application of vaccination across different dam populations remain poorly understood, especially in conditions where vaccination can be applied to the incoming and/or recipient dams to promote decreased shedding of the bacterium. In infected or naïve populations, the impact of one or two-dose vaccinations on *M. hyopneumoniae* infection and/or transmission has been evaluated. With one vaccination, a numerical reduction in *M. hyopneumoniae* adjusted reproductive number was estimated for vaccinated groups (R_n=0.71-2.38) compared to non-vaccinated groups (R_n=0.56-3.51, p>0.05, Meyns et al., 2006; Villarreal et al., 2011). Moreover, Pieters et al. (2010) demonstrated that the vaccination of sentinels with two doses prior to housing with infected pigs did not prevent colonization, as the proportion of infected sentinels did not change based on vaccination status. Indirectly, this concept has been investigated by assessing colonization of piglets born to vaccinated dams (Sibila et al., 2008; Garza-Moreno et al., 2019). Nevertheless, the combined effect of multiple vaccinations in both populations (infected and susceptible pigs) has been minimally evaluated. Therefore, the aim of this pilot study was to assess the effect of multiple vaccinations on *M. hyopneumoniae* infection, transmission, and genetic variability in infected and susceptible gilt populations.

3.3 Materials and methods

3.3.1 Ethics statement

All animals were cared for according to protocols designed specifically for the project, which were approved by the Institutional Animal Care and Use Committee of the University of Minnesota. Handling of the *M. hyopneumoniae* inoculum was performed following protocols approved by the Institutional Biosafety Committee at the University of Minnesota.

3.3.2 Animals and housing

Thirty-two commercial gilts were randomly selected at 3 weeks of age from a *M. hyopneumoniae* and Porcine Reproductive and Respiratory Syndrome Virus negative wean-to-finish farm, which was sourced from a negative sow herd. Prior to the study, their negative *M. hyopneumoniae* status was confirmed via the absence of seropositivity and detection of *M. hyopneumoniae* genetic material using blood and tracheal samples, respectively. Six weeks into the study, gilts were transported and housed in a filtered, accredited biosafety level-2 isolation unit at the University of Minnesota.

3.3.3 Experimental design

A graphic representation of the study experimental design is provided in Figure 3.1. The study consisted of three phases, namely: vaccination, seeder development, and transmission period. Gilts were randomly allocated to four treatment groups at the beginning of the vaccination phase (n=8/group): 1) Vaccinated seeder (VS); 2) Non-vaccinated seeder (NVS); 3) Vaccinated contact (VC); and 4) Non-vaccinated contact (NVC). Once allocated, gilts were housed by treatment group. The sample size for each group was calculated based on the ability to detect significant differences in number of infections between seeder and susceptible contact gilts across treatment groups using a

0.05 error rate. A brief description of the research procedures conducted during each study phase is presented as follows:

3.3.3.1 Vaccination

Gilts to be vaccinated (VS n=8; VC n=8) received 1 mL of a commercial *M. hyopneumoniae* bacterin intramuscularly (Ingelvac Mycoflex®, Boehringer Ingelheim Vetmedica GmbH, used under license) at 5, 7, and 9 weeks of age, for a total of three doses. At each vaccination event, blood samples were collected from all gilts in the study (n=32) to evaluate seroconversion.

3.3.3.2 Seeder development

For the purpose of this study, seeders were defined as gilts that were experimentally inoculated with *M. hyopneumoniae* and were confirmed infected at 28 days post-inoculation (dpi). At 11 weeks of age, eight VS and eight NVS gilts were transported to the isolation unit three days prior to inoculation for acclimation, and were housed in separate rooms by vaccination status. Tracheal samples and blood samples were collected from each gilt at arrival. At 0 dpi, gilts (n=16) were intra-tracheally inoculated with 10 mL of a lung homogenate containing $1x10^5$ CCU/mL of *M. hyopneumoniae* strain 232 (obtained from Iowa State University, Ames, IA, USA), which has been fully sequenced (Minion et al., 2004) and known to be of moderate virulence. The inoculation technique was performed without sedation and as described by Gomes Neto et al. (2014), using a sterile rubber urinary catheter (Covidien, Mansfield, MA, USA). At 28 dpi, tracheal samples and blood samples were collected from each

individual gilt to confirm infection. Afterwards, seeder gilts (VS & NVS) were relocated to clean, individual experimental rooms (n=16).

3.3.3.3 Transmission period

Each transmission period consisted of 14 days of contact between one seeder and one susceptible contact gilt for a total of four replicates per seeder-to-contact treatment group pairing. In doing so, the following seeder-to-contact treatment group pairings were evaluated: NVS:NVC, VS:NVC, NVS:VC, VS:VC. Seeder gilts were housed with susceptible contact gilts at peak of shedding (Roos et al., 2016). Prior to introduction with seeders, tracheal samples and blood samples were collected from each contact gilt to confirm negative *M. hyopneumoniae* infection status. To assess *M. hyopneumoniae* transmission, tracheal samples and blood samples were collected from all gilts at 7 and 14 days post-contact (dpc). Throughout the sampling, investigators were blinded as to which gilts were the seeders or the contacts.

3.3.4 Sample collection and processing

Tracheal samples were collected via intra-tracheal introduction of a sterile rubber urinary catheter (Covidien, Mansfield, MA, USA) with the aid of a laryngoscope and mouth speculum (Fablet et al., 2010). Tracheal secretions were obtained by washing tip of catheter with 500uL of phosphate-buffered saline. Sampling equipment was disinfected with 70% ethanol and rinsed with water between gilts to avoid sample cross-contamination. Blood samples were collected from the jugular vein of each gilt using vacutainer tubes (BD Vacutainer® Blood Collection Tubes, Franklin Lakes, NJ, USA) and sterile needles (BD Vacutainer® Blood Collection Needles, Franklin Lakes, NJ,

USA). Prior to processing, tracheal samples and blood samples were stored at -20°C and 4°C, respectively.

At euthanasia, the percentage of anatomic lung lobe lesions was recorded by a masked investigator, as previously described by Pointon et al. (1999). Additionally, a bronchial swab was collected from each gilt by sampling a bronchiole of grossly affected, apical or cardiac lung lobes with a sterile rayon swab (BBL CultureSwab™, Copan Italia Spa, Brescia, Italy). If macroscopic lesions were absent, a pre-determined lobe was sampled. Microscopic lung lesions were scored based on a modified scoring system from Calsamiglia et al. (2000b), which included a 5th score when large parabronchial lymphoid nodules were present with absence of alveolar inflammation.

Samples were individually tested at the University of Minnesota Veterinary Diagnostic Laboratory. Detection of the microorganism in tracheal samples and bronchial swabs was achieved using a *M. hyopneumoniae* real-time PCR (Strait et al., 2008). Furthermore, the presence of *M. hyopneumoniae* antibodies was assessed using a blocking ELISA (Dako Corporation, Carpinteria, CA, USA) in serum samples.

3.3.5 Diagnostic interpretation

Tracheal samples and bronchial swabs were considered positive with a <40 Ct value. At the end of the transmission period, gilts were determined to be *M. hyopneumoniae* positive based on a parallel interpretation of diagnostic results. This diagnostic criterion was used to evaluate *M. hyopneumoniae* transmission across treatment groups. Using a blocking ELISA, seroconversion was evaluated by comparing the sample optical density (OD) value to the OD value of the buffer control, yielding a

percent inhibition value. A sample was considered positive if the percent inhibition of the sample was ≤ 50% of the buffer control.

3.3.6 Multiple-locus Variable Number tandem repeat Analysis (MLVA)

To assess the effect of *M. hyopneumoniae* vaccination on genetic variability, MLVA was performed on *M. hyopneumoniae* positive tracheal samples and bronchial swabs to obtain a Variable Number Tandem Repeat (VNTR) type. The procedure was performed as described by Dos Santos et al. (2015), in which two loci, namely P97 and P146, were targeted. For assay verification, the *M. hyopneumoniae* ATCC 25095 reference strain was used as a positive control. The number of tandem repeats within each loci was estimated using a bioinformatics analytic software (BioNumerics, version 7.1, Applied Maths, Austin, TX, USA). Similar parameters described by Dos Santos et al. [36] were used for the determination of VNTR types.

3.3.7 Data analysis

Differences in relative bacterial load (Ct values) and seroconversion based on seeder gilt vaccination status were assessed using a Welch two sample t-test. Furthermore, a non-parametric Mann-Whitney t-test and Pearson's Chi-square test were utilized to compare percent of lung lesion and microscopic scores based on vaccination status of seeders and contact gilts, respectively. Diagnostic results, including those negative for detection of *M. hyopneumoniae* or antibodies, were included in the analysis. Results with a *p*-value <0.05 were deemed significant. By employing a maximum likelihood estimator (Bertram et al., 2018), the daily force of infection (λ) for each treatment group pairing was estimated. The probability that the contact gilts became

infected was calculated using the equation (Bertram et al., 2018): *Prob (transmission)= 1-e*$^{-\lambda T}$, in which a constant force of infection (λ) was assumed and *T* is the time (days) at which a contact gilt became *M. hyopneumoniae* positive during the transmission period. In addition, incidence rate based on pig-days at risk was calculated using *epitools* package (R Core Team, 2019). Data analyses were performed using R 3.6.1 (R Core Team, 2019).

3.4 Results

3.4.1 Vaccination

All gilts were serologically negative at the beginning of the vaccination phase. A decrease in percent inhibition was identified at the second vaccination in all VS and VC gilts (68.74±10.66) compared to NVS and NVC gilts (84.50±6.32; $p<0.01$; Figure 3.2), indicating a higher antibody response in vaccinated gilts compared to non-vaccinated gilts. Antibodies were detected in all tested VS and VC gilts at the third vaccination, while NVS and NVC gilts remained negative. Lower percent inhibition values were detected in VS and VC gilts (23.72±8.72) compared to NVS and NVC gilts (88.47±5.99; $p<0.01$) at the end of the vaccination phase, suggesting a high antibody response. *Mycoplasma hyopneumoniae* antibodies could not be evaluated in one VS gilt due to insufficient sample volume.

3.4.2 Seeder development

Prior to inoculation (0 dpi), all VS and NVS gilts (n=16 total) were *M. hyopneumoniae* negative by real-time PCR. *Mycoplasma hyopneumoniae* seroconversion was confirmed in all tested VS gilts, whereas all tested NVS gilts remained seronegative.

For two VS gilts, the presence of *M. hyopneumoniae* antibodies could not be evaluated due to insufficient sample volume.

At the end of the seeder development phase (28 dpi), *M. hyopneumoniae* was detected by PCR in 75% (6/8) and 100% (8/8) of the VS (29.71±6.8) and NVS (26.17±3.3) gilts, respectively. Differences in *Mycoplasma hyopneumoniae* Ct values between groups were not statistically significant, regardless of seeder vaccination status (p=0.25). Moreover, 100% (8/8) and 87.5% (7/8) of the VS and NVS gilts were seropositive for the microorganism. Lower percent inhibition values were detected in VS gilts (6.65±9.2) compared to NVS gilts (39.17±9.8; p<0.01).

3.4.3 Transmission period

3.4.3.1 Detection of M. hyopneumoniae genetic material

Antemortem detection of *M. hyopneumoniae* in seeders and their corresponding contact gilts based on treatment group pairing is shown in Figure 3.3. Upon arrival to the isolation facility, all contact gilts (NVC and VC) were negative for *M. hyopneumoniae.* Throughout the transmission period, all NVS gilts in the NVS:NVC treatment group pairing were *M. hyopneumoniae* positive. At 14 dpc, 25% (1/4) of the NVC gilts became infected with *M. hyopneumoniae.*

In comparison, all VS gilts in the VS:NVC treatment group pairing became *M. hyopneumoniae* positive at the transmission period (Figure 3.3B). Additionally, half (2/4) of the NVC gilts became infected with *M. hyopneumoniae* at the end of the transmission period. For the NVS:VC treatment group pairing, all NVS gilts were *M. hyopneumoniae* positive throughout the transmission period. At 7 dpc, 25% (1/4) of the VC gilts became

infected with *M. hyopneumoniae*, which remained positive at 14 dpc (Figure 3.3C). In the VS:VC treatment group pairing, all VS and VC gilts were *M. hyopneumoniae* positive and negative, respectively, throughout the transmission period (Figure 3.3D).

Based on seeder vaccination status, a reduction in *M. hyopneumoniae* relative bacterial load was detected in VS gilts (33.22±4.8) compared to NVS gilts (27.45±2.8; p=0.01) at 7 dpc. Moreover, significant differences in *M. hyopneumoniae* Ct values were also detected in VS gilts (34.97±4.1) and NVS gilts (30.05±3.1; p=0.02) at the end of the transmission period (14 dpc).

Post-euthanasia, 100% (16/16) of the seeders were *M. hyopneumoniae* positive in bronchial swabs regardless of vaccination status. Mean Ct values were numerically higher for VS gilts (29.3±6.1) compared to NVS gilts (27.8±2.9; p=0.58). Table 3.1 provides postmortem diagnostic results in contact gilts. At 14 dpc, two NVC gilts in the VS:NVC treatment group pairing became *M. hyopneumoniae* positive in bronchial swab. Moreover, one NVC gilt and one VC gilt in the VS:NVC and VS:VC treatment group pairings was *M. hyopneumoniae* positive in bronchial swabs. All bronchial swabs collected from VC gilts in the VS:VC treatment group pairing remained negative (Table 3.1). Cycle threshold values for all PCR testing are provided in Table S1.

3.4.3.2 Detection of M. hyopneumoniae antibodies

Antibody levels were higher for VS gilts compared NVS gilts at 0 and 7 dpc (p<0.01). At 14 dpc, 75% (6/8) and 100% (8/8) of the VS and NVS gilts, respectively, were seropositive. Differences in antibody levels between the two groups were not significant (p=0.19) at 14 dpc. At 0 dpc, all VC gilts (8/8) were seropositive. Over time, a

numerical decrease in the number of positive samples was detected in VC gilts, in which 87.5% (7/8) and 62.5% (5/8) remained seropositive at 7 and 14 dpc, respectively. All NVC gilts (8/8) remained seronegative for *M. hyopneumoniae* throughout the entire transmission period. For detailed information regarding antibody levels for all gilts, refer to Table S1.

3.4.3.3 Lung lesion scores

Mean macroscopic lung lesions (%) were numerically lower in VS gilts (2.87±2.99) compared to NVS gilts (4.12±5.14, p=0.78). Differences in microscopic scores based on seeder gilt vaccination status were not detected (p=0.80). Macroscopic and microscopic lung lesions were not identified in any of the VC gilts from the VS:VC treatment group pairing, compared to the other pairings (Table 3.1).

3.4.3.4 Transmission parameters

Numerical differences in λ and incidence rate were estimated between the four treatment group pairings (Table 3.2). The mean rate at which a contact gilt became infected per day and incidence rate was lowest when both seeder and contact gilts were vaccinated.

3.4.4 MLVA typing

At total of 66 *M. hyopneumoniae* PCR positive samples were processed for MLVA typing. Throughout the entire study, a single MLVA type (14-21) was detected in both VS and NVS gilts and their corresponding *M. hyopneumoniae* positive, contact gilts (data not shown).

3.5 Discussion

The effect of multiple vaccinations on *M. hyopneumoniae* infection, transmission, and genetic variability in infected and susceptible gilt populations was assessed in this study. Administration of three vaccine doses prior to *M. hyopneumoniae* infection resulted in the development and persistence of antibodies throughout the study, as well as the reduction in *M. hyopneumoniae* relative bacterial load in vaccinated seeder gilts compared to non-vaccinated seeder gilts. Although complete protection to *M. hyopneumoniae* infection was not evident, vaccination of both seeders and their corresponding contact gilts numerically reduced the rate at which contact gilts became infected, resulting in a lack of transmission during a two-week contact period. In comparison, at least one contact gilt became infected with *M. hyopneumoniae* for the remaining treatment group pairings. Differences in *M. hyopneumoniae* genetic variability due to vaccination were not observed as a single VNTR type was detected in all *M. hyopneumoniae* positive samples in this study.

It has been suggested that *M. hyopneumoniae* transmission may be reduced using vaccines due to the known effects that this control strategy has on lowering bacterial loads (Meyns et al., 2008; Pieters et al., 2010; Villarreal et al., 2011). To best address this question, three vaccine doses were administered to induce a strong immune response and reduce *M. hyopneumoniae* colonization, thus optimal conditions for inhibition of transmission could be achieved. In the present study, a reduction in *M. hyopneumoniae* relative bacterial load was evident in vaccinated seeder gilts compared to non-vaccinated seeder gilts. This finding is supportive of previous work in which similar results have been observed (Baccaro et al., 2006; Sibila et al., 2007a; Woolley et al., 2014). Vaccine

timing and the number of doses are thought to be influential for reducing *M. hyopneumoniae* infection. Traditionally, the maximum beneficial effects for *M. hyopneumoniae* control post-vaccination have been proposed to occur several months after implementation (Haesebrouck et al., 2004). However, studies specifically looking at this topic are scarce. In this study, the timing of the first vaccination occurred six weeks prior to inoculation, thus allowing enough time for gilts to develop a strong immune response to reduce clinical severity and disease outcomes. The onset of serological response was detected four weeks after the first vaccination, which has been similarly observed (Meyns et al., 2006; Baccaro et al., 2006). *Mycoplasma hyopneumoniae* antibodies were still detected in a majority of the vaccinated contact gilts eight weeks after the initial vaccination. Antibody levels have been detected until 1-3 months after vaccination (Maes et al., 2008). It is important to note that differences in onset and duration of serological response post-vaccination can vary among different vaccine preparations and number of vaccine doses. Garza-Moreno et al. (2019) observed numerical differences in the proportion of seropositive piglets and percentage of antigen inhibition within a sample when multiple vaccinations were administered to gilts, favoring four versus two doses.

In this study, lung lesion severity was not statistically different in vaccinated and non-vaccinated seeder or contact gilts. Reduction in lung lesions and cough severity, along with improved growth performance, are considered main outcomes from *M. hyopneumoniae* vaccination and have been used to measure vaccine efficacy (Sibila et al., 2007a; Maes et al., 1998). Differences in seeder lung lesion scores in this study were likely not observed due to a large variation in lesion severity within treatment groups,

thus reducing statistical power. Moreover, findings from a systematic review have described several factors, including study duration and *M. hyopneumoniae* strain, to be influential for the variation in disease severity (Garcia-Morante et al., 2017). In this study, lung lesion severity in seeders was assessed at 42 dpi, a time between when peak macroscopic lung lesions (28 dpi) and increased variation in lung lesion severity (56 dpi) have been described (Garcia-Morante et al., 2017). In other studies, similar or minimal differences in lung lesion scores between non-vaccinated and vaccinated pigs have also been suggested to result from an inadequate immune response prior to exposure (Maes et al., 1999; Sibila et la., 2007a) or a short infectious period. In the field, significant differences in macroscopic lung lesions between non-vaccinated and vaccinated (one or two-dose) pigs were not evident until 25 weeks of age, in which pigs were colonized with *M. hyopneumoniae* as early as three weeks of age (Sibila et al., 2007a). Under experimental conditions, Meyns et al. (2006) identified significant differences in lung lesions between non-vaccinated and vaccinated (one-dose) seeders and their naïve contact gilts at six weeks post-infection. In the mentioned investigations, a highly virulent strain and greater infectious dose were utilized, as opposed to this study in which a strain of moderate virulence and the minimal infectious dose (Marois et al., 2010) to induce pneumonia were employed. Pertaining to the contact gilts, the duration of transmission was likely too short (14 days) to observe significant differences in macroscopic lung lesions.

The ability to measure differences in transmission as the result of an intervention can be challenging to achieve as there can be several factors that affect the transmission dynamics. Within a population, the number of susceptible and infectious individuals, the

number of contacts, and the probability of transmission are all factors that can influence the transmission of a pathogen. Moreover, additional variables including but not limited to the stocking density, frequency of contacts, and population size etc., should be considered when designing a study. For this investigation, an experimental model that incorporated a 1:1 seeder-to-contact ratio was employed. By conducting this experiment in such manner, other factors that could be present under field conditions were minimized to provide further insight into the potential causality of vaccination on transmission. Moreover, the *M. hyopneumoniae* vaccine was administered to seeders, susceptible contact gilts, or both to best understand the full effect of this intervention on transmission. Another advantage of conducting an experimental model that incorporates a 1:1 seeder-to-contact ratio is that the likelihood of infection from other individuals can be ruled out, thus making the probability of infection more robust (Velthuis et al., 2002). Under experimental conditions, Pieters et al. (2010) and Meyns et al. (2006) evaluated the effect of two or one vaccine doses on *M. hyopneumoniae* transmission in only contact gilts or in both seeders and contacts, respectively. Moreover, Villarreal et al. (2011) investigated this topic further by conducting a field study, in which one vaccine dose was administered to seeders. Across all studies, significant differences in *M. hyopneumoniae* transmission based on vaccination were not observed. However, a numerical reduction in the *M. hyopneumoniae* adjusted reproductive number was estimated in vaccinated versus non-vaccinated groups (Meyns et al., 2006; Villarreal et al., 2011). The results of this present study are in agreement with previous studies that concluded vaccination to numerically reduce *M. hyopneumoniae* transmission during a short period of time. In this study, statistical differences were not found due to the

overlapping confidence levels across groups. However, small variations in the rate at which susceptible pigs become infected (λ) may theoretically result in major differences in the total number of infected gilts when longer time periods are considered. Considering different intra-herd dynamics in the field, there may be some biological importance to vaccinate incoming naive and infected recipient populations, such that the infectious pressure in the herd may be reduced over time. Routine administration of multiple vaccine doses in a population may not be as appealing when considering additional costs associated with labor and the vaccine product. However, the application for this study would be most advantageous in *M. hyopneumoniae* positive herds that undergo gilt acclimation or an elimination program, in which case the benefits may outweigh the costs associated with the intervention, if herd stability is achieved earlier. Further research encompassing larger populations across longer time periods is necessary to confirm such findings and potential implications. Additional research directed at improving vaccine efficacy would also be desired to aid in *M. hyopneumoniae* prevention.

Statistical differences in *M. hyopneumoniae* transmission were not observed, potentially due to a short contact period. Since the experimental period was much shorter than the infectious period and considering the relatively slow onset of *M. hyopneumoniae* infection, the influence of vaccination on transmission could be greater under conditions in which the effect of the intervention can be fully observed. Moreover, additional replicates would have helped to validate the findings in this study, such that the likelihood of these events occurring due to random chance could be ruled out. In addition, the two VS gilts that remained *M. hyopneumoniae* negative until 14 dpc may have altered the overall number of NVC gilts that became *M. hyopneumoniae* positive throughout the

transmission period. Such finding could have resulted from the intrinsic variability of sampling live pigs, the inoculation method, or a delay in onset of infection due to the vaccination. In this study, intra-tracheal inoculation was performed as this method has been described to result in a high proportion of pigs infected with *M. hyopneumoniae,* as well as significant lung lesions compared to other exposure routes (Garcia-Morante et al., 2016). However, variability in *M. hyopneumoniae* infection models has been described, even in conditions when the same strain and dose are utilized (Garcia-Morante et al., 2016).

Intra-herd genetic diversity of *M. hyopneumoniae* has been identified, primarily through the utilization of MLVA (Betlach et al., 2019). However, drivers for *M. hyopneumoniae* genetic diversity continue to remain unknown. Michiels et al. (2017) demonstrated a higher diversity of *M. hyopneumoniae* variants in bronchoalveolar lavage fluid collected from slaughter pigs sourced from vaccinated herds. Nevertheless, additional reseach is needed to further clarify this topic. It has been hypothesized that genomic modifications on surface adhesin proteins may result from pressures influencing host-pathogen adaptations (Zhang et al., 1995). The *M. hyopneumoniae* genome has several surface proteins that have been described to be involved in cilia adhesion and/or antigenic variation (Zhang et al., 1995; Assunção et al., 2005b). Antigenic variation is a defense mechanism in which pathogens can undergo alterations in immunogenic epitopes that help facilitate evasion of the host immune system and promote persistence of infection (van der Woude and Baumler, 2004). One of the targeted loci in MLVA, namely P97, is a *M. hyopneumoniae* surface adhesin that has been suggested to undergo antigenic variation (Zhang et al., 1995). To help investigate this hypothesis, the genetic

diversity of *M. hyopneumoniae* post-vaccination was investigated. Under the conditions of this study, a single VNTR type was detected in vaccinated and non-vaccinated gilts, which is the same VNTR type as the strain utilized in the study. The detection of a single VNTR type suggests that vaccine administration did not influence the genetic diversity of this pathogen post-infection. However, we can not rule out other factors, such as timing, that might have contributed to a limited genetic diversity. Additional reseach is needed to understand potential drivers for *M. hyopneumoniae* diversity, especially considering the very short duration of this study.

3.6 Conclusions

In conclusion, the present study showed that a three-dose vaccination strategy against *M. hyopneumoniae* significantly reduced the relative bacterial load in infected gilts. Furthermore, a numerical reduction in *M. hyopneumoniae* transmission and lung lesions in susceptible contact gilts was observed across seeder-to-contact pairings with differing vaccine dynamics, favoring conditions when both seeders and their corresponding contact gilts were vaccinated. This study provides additional insight on the potential application of multi-dose vaccination strategies for *M. hyopneumoniae* control in incoming and/or recipient populations.

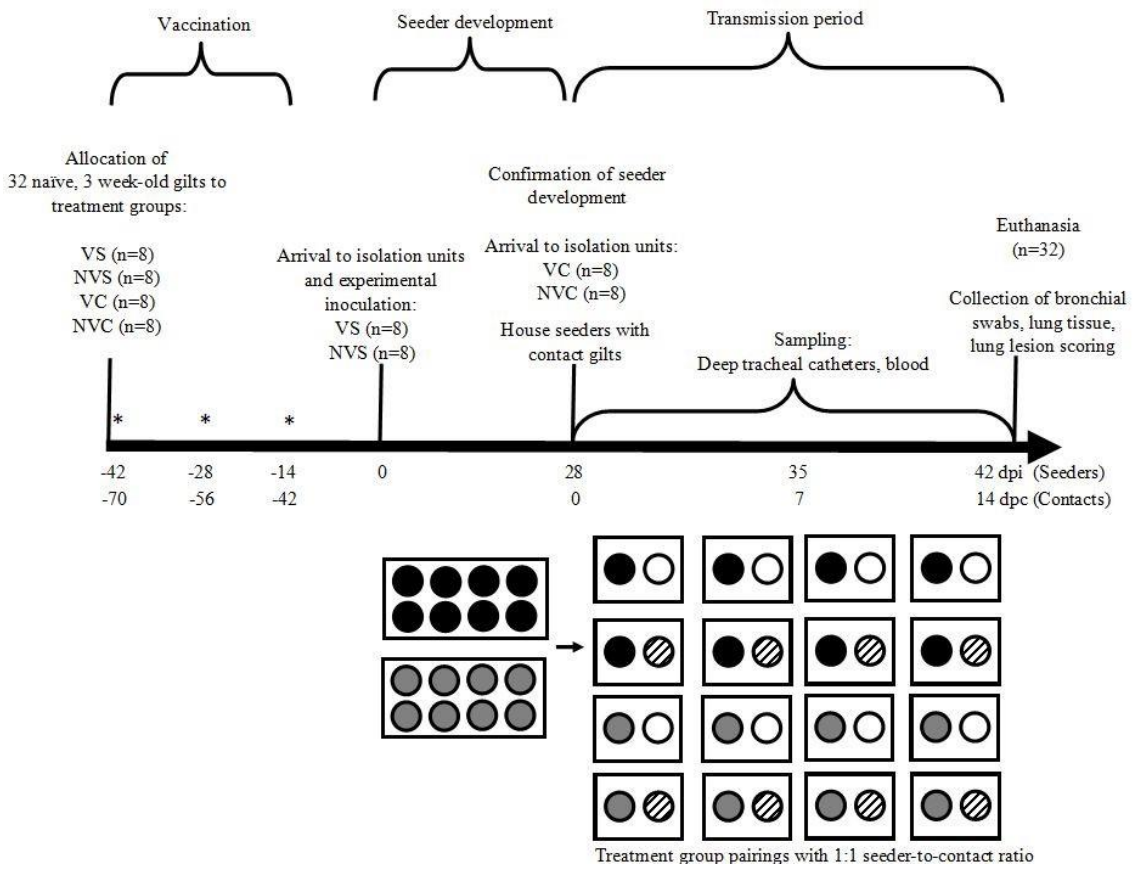

Figure 3.1 Schematic overview of study design

●=VS (Vaccinated seeder). ●=NVS (Non-vaccinated seeder). ○=VC (Vaccinated contact).

⊘=NVC (Non-vaccinated contact).

Each circle corresponds to an individual gilt and is color-coded based on treatment. Each box represents an individual experimental room. dpi=days post-inoculation. dpc=days post-contact. *=vaccination administered.

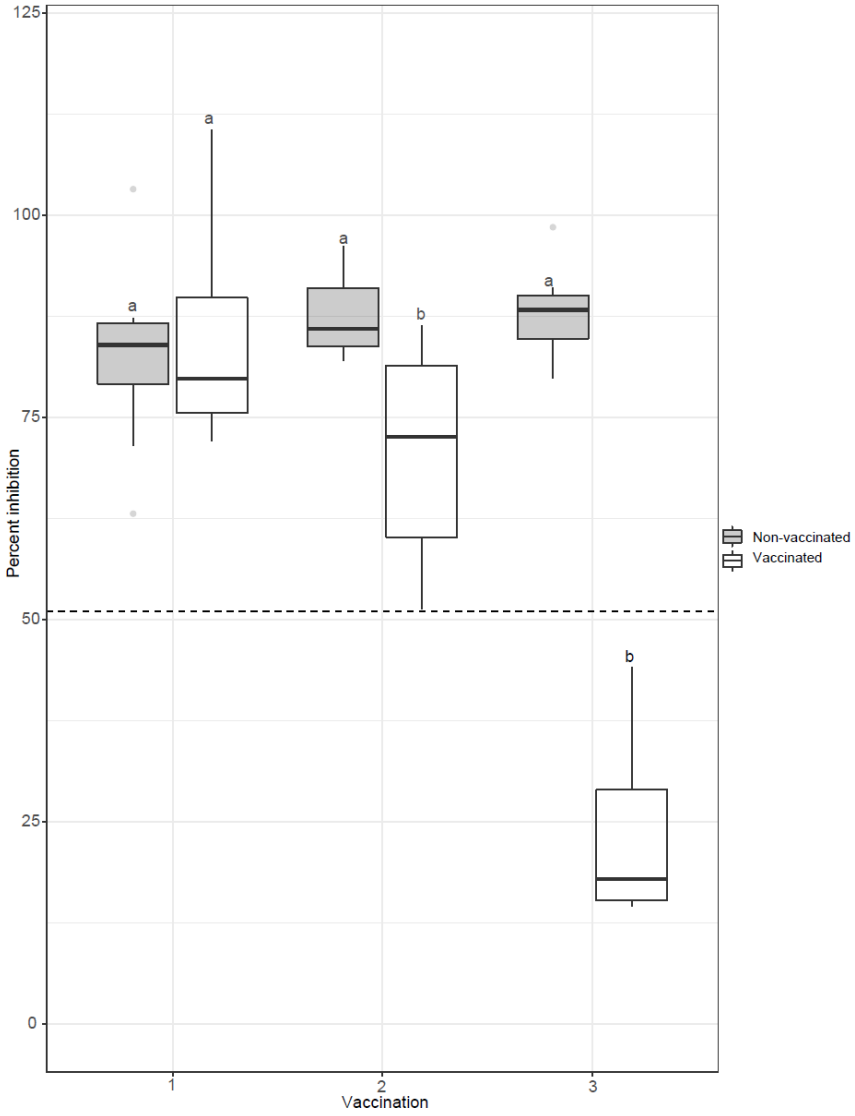

Figure 3.2 *Mycoplasma hyopneumoniae* seroconversion based on gilt vaccination status and timing

Grey and white colors correspond to non-vaccinated or vaccinated gilts, respectively. Different superscripts represent significant differences in mean OD value across groups.

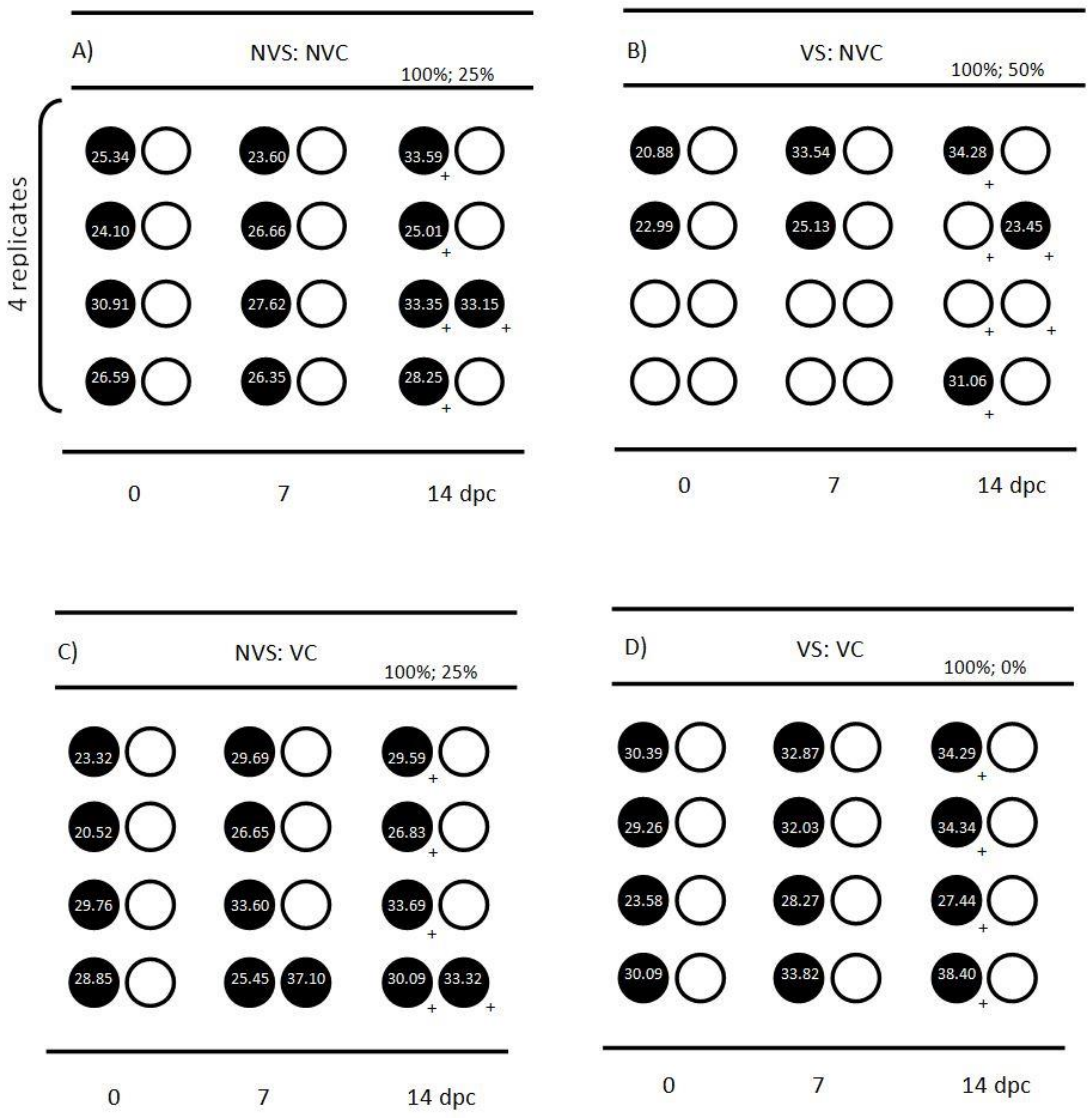

Figure 3.3 Detection of *Mycoplasma hyopneumoniae* in seeders and their corresponding contact gilts based on treatment group pairing

Treatment: VS (Vaccinated seeder); NVS (Non-vaccinated seeder); VC (Vaccinated contact); NVC (Non-vaccinated contact). For each pairing, treatment of seeder is stated first followed by treatment of contact gilt. Each circle corresponds to an individual gilt. Black circles represent detection of *M. hyopneumoniae* in tracheal samples. In each black circle, Ct values for *M. hyopneumoniae* detection are present. White circles represent absence of detection. Symbol "+" represents detection of *M. hyopneumoniae* in bronchial swabs. Dpc=days post-contact.

Table 3.1 Summary of *Mycoplasma hyopneumoniae* diagnostic results in contact gilts based on corresponding seeder-to-contact treatment group pairing at 14 days post-contact

		Contact gilts (n=4/group)			
Treatment group pairing*		NVS: **NVC**	VS: **NVC**	NVS: **VC**	VS: **VC**
Tracheal samples	Number positive	1	1	1	0
	Ct value(s)	33.15	31.06	33.32	---
Bronchial swabs	Number positive	1	2	1	0
	Ct value(s)	29.49	36.52, 38.01	35.13	---
Macroscopic lung lesions	Mean score (min, max)	0.50 (0,2)	1.50 (0,3)	1.00 (0,3)	0 (0,0)
Microscopic lung lesions	Mean score (min, max)	1.75 (0,4)	3.25 (0,5)	2.25 (0,5)	0 (0,0)

Treatment: VS (Vaccinated seeder); NVS (Non-vaccinated seeder); VC (Vaccinated contact); NVC (Non-vaccinated contact). For each pairing, treatment of seeder is stated first followed by contact gilt treatment. *Data represents diagnostics results and lung lesions of only contact gilts (NVC and VC), which is presented according to seeder-to-contact treatment group pairing.

Table 3.2 *Mycoplasma hyopneumoniae* transmission parameters based on seeder-to-contact treatment group pairing

Treatment group pairing	Force of infection (95% CI)	Incidence rate (95% CI)
NVS: NVC	0.021 (0.001, 0.091)	0.018 (0.005, 0.099)
VS: NVC	0.050 (0.009, 0.159)	0.034 (0.004, 0.129)
NVS: VC	0.022 (0.001, 0.097)	0.020 (0.002, 0.114)
VS: VC	2.30E-11 (0.000, 0.034)	0.000 (0.000, 0.066)

Treatment: VS (Vaccinated seeder); NVS (Non-vaccinated seeder); VC (Vaccinated contact); NVC (Non-vaccinated contact). For each pairing, treatment of seeder is stated first followed by contact treatment.

Chapter 4: Effect of antibiotic treatment on *Mycoplasma hyopneumoniae* detection and infectious potential

Work from this chapter has been accepted for publication in the journal **Veterinary Microbiology.**

4.1 Summary

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) causes significant economic losses in the swine industry. Antibiotics with activity against *Mycoplasma spp.* are employed for disease mitigation and pathogen elimination. However, veterinarians are often challenged with the detection of *M. hyopneumoniae* by PCR after antibiotic treatment, thus raising the question whether the bacterium is still infectious. The objective of this study was to evaluate the effect of tulathromycin treatment on *M. hyopneumoniae* detection and infectious potential during the acute and chronic phases of infection. For each infection phase, one age-matched naïve gilt was placed in contact with one *M. hyopneumoniae* infected gilt that was either treated with tulathromycin, treated and vaccinated, or non-treated, for 14 days. Four replicates per treatment group were performed for each infection phase. A numerical reduction in relative bacterial load was observed in acutely, treated gilts compared to non-treated gilts. The rate at which naïve gilts became infected with *M. hyopneumoniae* was numerically reduced when co-housed with treated, acutely infected gilts compared to those housed with non-treated, infected gilts. During the chronic infection phase, *M. hyopneumoniae* was detected by PCR in more than 50% of treated infected gilts and persisted for up to three months post-treatment. In addition, transmission was not detected in all treatment groups, however, the possibility that the pathogen was infectious could not be completely ruled out. Further research focused on assessing *M. hyopneumoniae* detection and viability post-treatment is necessary to guide control and elimination efforts.

4.2 Introduction

Infections caused by *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) are prevalent worldwide and continue to be one of the most significant bacterial health concerns in swine (Pieters and Maes, 2019). The effect of *M. hyopneumoniae* infection is mainly tangible in altered finishing production parameters, such as decreased average daily gain and poor feed efficiency. It has been suggested that controlling *M. hyopneumoniae* infection during the finishing period is primarily influenced by the health status of the sourcing breeding herd(s), as weaned piglets are often colonized from shedding dams (Calsamiglia and Pijoan, 2000a). After colonization, *M. hyopneumoniae* can be shed for a long period of time (at least up to 214 days post-inoculation; Pieters et al., 2009), resulting in infection and clinical disease during the grower-finisher phase. Moreover, a positive correlation between *M. hyopneumoniae* prevalence at weaning and disease severity at marketing has been shown (Fano et al., 2007). To combat *M. hyopneumoniae* infection, control and elimination strategies are often implemented in the field. Changes in management practices, vaccination, and antibiotic treatment are used, alone or in combination, to mitigate the detrimental effects of *M. hyopneumoniae* infection in swine herds. In practice, antibiotic treatments with activity against *Mycoplasma spp.* are commonly employed for disease mitigation, as well as for pathogen elimination (Maes et al., 2018).

Tulathromycin is a macrolide highly effective for treating respiratory disease in swine (Nutsch et al., 2005) and is commonly employed for *M. hyopneumoniae* disease elimination in the field (Holst et al., 2015). Moreover, immune-modulatory properties of tulathromycin have been shown to aid in the resolution of inflammation in the context of respiratory disease in pigs (Duquette et al., 2015). Following antibiotic treatment, it is

essential to accurately assess changes in disease outcome and evaluate pathogen clearance to guide health and management decisions. However, this can be challenging as *M. hyopneumoniae* genetic material has been detected by PCR up to 189 days post-inoculation, even after tulathromycin treatment has been applied (Painter et al., 2012). Persistence of *M. hyopneumoniae* detection post-treatment with other classes of antibiotics, such as tetracyclines and fluoroquinolones, has also been described (Thacker et al., 2006; Le Carrou et al., 2006). Although antibiotic resistance of *M. hyopneumoniae* has been shown to a limited extent (Maes et al., 2020b), the continued detection of genetic material by PCR in treated pigs could result from its slow degradation and clearance by immune cells and mechanical defenses of the respiratory system. However, the infectious potential of the bacterium under these conditions has not been investigated.

In addition, the significantly low sensitivity of bacterial culture for *M. hyopneumoniae* is a limitation to evaluate pathogen viability *in vitro* (Sibila et al., 2009). Thus, the research question of the infectious capability of *M. hyopneumoniae* genetic material detected by PCR in treated pigs remains to be answered. Therefore, the objective of this study was to evaluate the effect of tulathromycin on *M. hyopneumoniae* detection and infectious potential during the acute and chronic phases of infection using an *in vivo* transmission model.

4.3 Materials and methods

4.3.1 Animals, housing, and ethics statement

A total of 48 gilts from a *M. hyopneumoniae* and Porcine Reproductive and Respiratory Syndrome Virus negative farrow-to-finish farm were randomly selected and ear-tagged at three weeks of age. Negative status of the farm was based on several years

of herd diagnostic results, the absence of *M. hyopneumoniae* detection by PCR and seroconversion, and the lack of suggestive clinical signs. Three days prior to inoculation, 24 3-week-old gilts were transported to experimental isolation units at the University of Minnesota and housed together by treatment group for acclimation. An additional 24 naïve gilt were later incorporated in the study and housed with infected gilts during the acute and chronic infection phases. At arrival, gilts were confirmed negative to *M. hyopneumoniae* by the absence of clinical signs suggestive of infection, lack of pathogen detection in tracheal secretions, and lack of seroconversion to the bacterium. All gilts were cared for according to project-specific protocols approved by the Institutional Animal Care and Use Committee, University of Minnesota. Use and handling of the *M. hyopneumoniae* inoculum were approved by the Institutional Biosafety Committee of the University of Minnesota.

4.3.2 Experimental design

The experimental design of this study is depicted in Figure 4.1. The study was composed of four phases: 1) Infection development, 2) Transmission assessment during acute infection phase, 3) Transmission assessment during chronic infection phase, and 4) Persistence post-treatment. Gilts were randomly allocated to one of seven treatment groups: Naïve contact gilts for acute and chronic infection phases (n=24); Acutely infected, control (AC, n=4); Acutely infected and treated (AT, n=4); Acutely infected, treated and vaccinated (ATV, n=4); Chronically infected, control (CC, n=4); Chronically infected and treated (CT, n=4); and Chronically infected, treated and vaccinated (CTV, n=4). Power analysis was not employed to estimate sample size, as this was a pilot study. Once allocated, gilts were housed together by treatment group until the start of the

transmission experiments. Briefly, two sets of transmission experiments, one during acute infection (28-42 days post-inoculation (dpi)) and one during chronic infection (85-99 dpi), were performed in this study. For each transmission experiment, one age-matched naïve gilt was housed with one *M. hyopneumoniae* infected gilt that was either non-treated (AC or CC), treated (AT or CT), or vaccinated and treated (ATV or CTV) for 14 days. Four replicates per treatment group per phase of infection were performed. All gilts that took part in the acute infection phase were euthanized at the end of the transmission experiment (42 dpi). For the chronic infection phase, all gilts were euthanized at the end of the transmission experiment (99 dpi), except for CT and CTV gilts that were *M. hyopneumoniae* positive by PCR and did not transmit the bacterium to their corresponding naive gilt. The persistence of *M. hyopneumoniae* detection post-treatment was assessed in the remaining CT and CTV gilts for an additional 56 days (99-155 dpi). Research procedures conducted during each study phase are described below:

4.3.3 Study phases

4.3.3.1 Infection development

Gilts in AC, AT, ATV, CC, CT, and CTV groups were intratracheally inoculated (Gomes Neto et al., 2014) with ten mL of a lung homogenate containing $1x10^5$ color-changing units (CCU)/mL of *M. hyopneumoniae* strain 232 (purchased from Iowa State University, Ames, IA, USA) for two consecutive days. Tracheal secretions were collected at 14 dpi from inoculated gilts to confirm infection via PCR detection of *M. hyopneumoniae.* Following inoculation, AC, AT, and ATV gilts were housed by treatment group until 28 dpi to develop acute *M. hyopneumoniae* infection. Chronically

infected, control (CC), CT, and CTV gilts were housed by treatment group for a longer period (until 85 dpi) to become chronically infected.

4.3.3.2 Transmission assessment during acute infection phase

At 8 and 18 dpi, AT and ATV gilts (n=4/group) were intramuscularly treated with 2.5 mg/kg of tulathromycin (Draxxin®, Zoetis, Parsippany, NJ, USA). Additionally, ATV gilts (n=4) were intramuscularly vaccinated with 2mL of a commercial *M. hyopneumoniae* bacterin (Respisure®, Zoetis, Parsippany, NJ, USA) at 0 and 23 dpi. At 28 dpi, gilts in AC, ACT, and ATV groups (n=4/group) were individually relocated to clean, experimental rooms, where they were housed with one age-matched naive gilt for 14 days. The naive gilts were placed in contact with AC, AT, and ATV gilts when peak of shedding has been shown under experimental conditions (Roos et al., 2016). Prior to housing with infected gilts, the health status of naive gilts was confirmed via the absence of *M. hyopneumoniae* detection by PCR and the absence of seroconversion. To assess *M. hyopneumoniae* transmission, tracheal secretions were collected at 0, 11, and 14 days post-contact (dpc) from all gilts. In addition, blood samples were collected at 0 and 14 dpc. Throughout sampling, investigators were masked as to which gilts were infected or naïve. At the end of the transmission experiment, all gilts were euthanized by barbiturate overdose, followed by the scoring of lung lesions and the collection of bronchial swabs and lung tissue.

4.3.3.3 Transmission assessment during chronic infection phase

During the chronic infection phase, CT and CTV gilts (n=4/group) were intramuscularly treated with 2.5 mg/kg of tulathromycin at 64 and 74 dpi. Furthermore,

CTV gilts were vaccinated with 2mL of the commercial *M. hyopneumoniae* bacterin at 0 and 23 dpi. Before the transmission experiment, one CC gilt and one CTV gilt died due to secondary bacterial infections. At 85 dpi, all remaining CC, CT, and CTV gilts (n=10 total) were relocated to individual, clean, experimental rooms and each gilt was housed with one naive gilt for 14 days. Tracheal secretions were collected at 0, 11, and 14 dpc, along with the collection of blood samples at the beginning and end of the transmission experiment. All gilts were euthanized at the end of the transmission experiment, except for CT and CTV gilts that were *M. hyopneumoniae* positive by PCR and did not transmit the bacterium to their corresponding naive gilt. Post-euthanasia, lung lesions were scored along with the collection of bronchial swabs and lung tissue.

4.3.3.4 Persistence of Mycoplasma hyopneumoniae post-treatment

To evaluate the persistence of *M. hyopneumoniae* detection by PCR post-treatment, a fourth phase of the study was conducted. Based on a diagnostic criterion, CT and CTV gilts that were *M. hyopneumoniae* PCR positive at the end of the chronic transmission experiment and did not transmit the bacterium to their corresponding naive gilt were kept for an additional 56 days (i.e., 99 to 155 dpi) for further sampling. A lack of transmission to naïve gilts was determined based on the absence of *M. hyopneumoniae* detection in the respective gilt by PCR. During the additional 56 days, CT and CTV gilts were housed by treatment group and tracheal secretions were collected from each gilt at approximately every two weeks. At 155 dpi, gilts were humanely euthanized. In addition, lung lesions were scored and bronchial swabs were collected in all gilts.

4.3.4 Sample collection and processing

Tracheal secretions were collected as described by Fablet et al. (2010) by introducing a sterile rubber urinary catheter (Covidien, Mansfield, MA, USA) into the mouth of each gilt until the trachea was reached. The tip of the catheter was placed into a sterile 5mL tube containing 500uL of phosphate-buffered saline and was vortexed. To minimize cross-contamination, handling equipment was disinfected with 70% ethanol and rinsed with water between each sampled gilt. Blood samples were collected from the jugular vein using vacutainer tubes (BD Vacutainer® Blood Collection Tubes, Franklin Lakes, NJ, USA) and sterile needles (BD Vacutainer® Blood Collection Needles, Franklin Lakes, NJ, USA). Tracheal secretions and blood samples were stored at -20^{o}C and 4^{o}C, respectively, until processed.

At euthanasia, macroscopic lung lesions were assessed and scored based on the percent of anatomical lobe affected (Pointon et al., 1999). Lesions were scored in all gilts by the same investigator, who was blinded to the pig identification throughout the process. Bronchial swabs were collected from each gilt by inserting a sterile rayon swab (BBL CultureSwab™, Copan Italia Spa, Brescia, Italy) into the main airways of the apical or cardiac lung lobe when macroscopic lung lesions were evident. If lesions were absent, a pre-determined area was systematically sampled. To evaluate microscopic lesions, lung samples were collected in the transitional area when lesions were evident. or in a consistent anatomical area if absent and submitted for histopathologic evaluation. Tissue samples were fixed in 10% formalin, stained with hematoxylin and eosin, and scored. The scoring system employed was slightly modified from Calsamiglia et al. (2000b), as the addition of a 5th score was included when lesions characterized by large

parabronchial lymphoid nodules with an absence of alveolar inflammation were present. Furthermore, a section of lung tissue was collected for bacterial aerobic culture.

All samples were submitted for individual testing at the University of Minnesota Veterinary Diagnostic Laboratory. Species-specific real-time PCR (Strait et al., 2008) was performed on tracheal secretions and bronchial samples to detect *M. hyopneumoniae* genetic material. For seroconversion assessment, blood samples were processed for serum separation and *M. hyopneumoniae* ELISA (Idexx, Westbrook, Maine, USA) was performed on serum.

4.3.5 Diagnostic interpretation

Samples were considered positive for detection of *M. hyopneumoniae* when a Ct value was <37. Samples with Ct values ranging from 37 to 40 were considered suspect for *M. hyopneumoniae.* Parallel interpretation of PCR results was used to determine the infection status of gilts, thus if either the tracheal secretions or bronchial swabs resulted PCR positive, the gilt was considered infected. For *M. hyopneumoniae* ELISA, samples were considered positive with a ≥ 0.4 S/P value.

4.3.6 Data analysis

Differences in *M. hyopneumoniae* detection and disease severity were assessed using descriptive statistics. Mean Ct values were calculated using positive samples and were used to assess the relative bacterial load within the lower respiratory tract. To assess differences in *M. hyopneumoniae* transmission, the daily force of infection (λ), the rate at which naive pigs became infected per day, was calculated using maximum likelihood estimator (Bertram et al., 2018). Throughout the transmission period, the probability of

infection was calculated using the equation, $Prob\ (transmission) = 1 - e^{-\lambda T}$ (Bertram et al., 2018) and λ was assumed to be constant. The incidence rate based on pig-days at risk was calculated by employing 'epitools' package (R Core Team, 2019). Data analyses were conducted using R 3.6.1 (R Core Team, 2019).

4.4 Results

4.4.1 Infection development

Upon arrival to the isolation facility, gilts tested negative for *M. hyopneumoniae* infection. At 14 dpi, all inoculated gilts (24/24) were confirmed *M. hyopneumoniae* positive with a mean Ct value of 24.1±2.9. A numerical decrease in the mean *M. hyopneumoniae* relative bacterial load was detected in AT gilts (26.1±2.3) at 14 dpi compared to AC gilts (22.6±1.6). In ATV gilts, a numerical reduction in the mean relative bacterial load (24.8±2.4) was also identified to a lesser degree.

4.4.2 Transmission assessment during acute infection phase

All infected gilts remained positive for *M. hyopneumoniae* throughout the transmission experiment, regardless of receiving tulathromycin treatment. At 0, 11, and 14 dpc, a numerical reduction in relative bacterial load was evident in AT gilts compared to other treatment groups (Table 4.1). At 0 dpc, a delay in seroconversion was detected in AT gilts compared to gilts in other treatment groups (Table 4.1).

Prior to housing with infected gilts, all naive gilts were negative for *M. hyopneumoniae.* At the end of the transmission experiment (14 dpc), 50% (2/4) of the naïve gilts that were housed with AC gilts became *M. hyopneumoniae* positive, in which one gilt was initially positive at 11 dpc (Table 4.1). In comparison, all naive gilts that

were housed with AT gilts remained negative during the transmission experiment. For the naive gilts housed with ATV gilts, 25% (1/4) became *M. hyopneumoniae* positive at 11 dpc.

Postmortem diagnostic results for AC, AT, and ATV gilts and their corresponding naive gilts, including macroscopic lung lesions and histopathologic scores, are presented in Table 2. Briefly, a numerical reduction in lung lesions (17.6-16.1%) was observed in AT and ATV gilts compared to AC gilts. Moreover, the relative bacterial load of *M. hyopneumoniae* was numerically lower in bronchial swabs collected from AT (21.4±1.7) and ATV (21.3±0.51) gilts compared to AC gilts (19.4±0.8; Table 4.2). Similar mean bronchial swab Ct values for AT and ATV gilts were detected. Using aerobic culture, *Glaesserella parasuis* was grown from lung tissue from one AC, one AT, and one naïve gilt (Table 4.2).

4.4.3 Transmission assessment during chronic infection phase

At the beginning and end of the transmission experiment, all CC gilts were positive for *M. hyopneumoniae* by PCR via tracheal secretions (Table 1). For the remaining treatment groups, *M. hyopneumoniae* was detected in a subset of the CT and CTV gilts (Table 1). At 14 dpc, a numerical reduction in the mean *M. hyopneumoniae* relative bacterial load was detected in CT gilts (32.2±0.7) and CTV gilts (30.4 ±5.2) compared to CC gilts (29.2 ±4.4). Fifty percent (2/4) of CT gilts and 33% (1/3) of CTV gilts were negative for *M. hyopneumoniae* in tracheal secretions at the end of the transmission experiment (Table 1). *Mycoplasma hyopneumoniae* antibodies were consistently detected during the chronic infection phase in all CC, CT, and CTV gilts (Table 4.1).

At 0 dpc, all naïve gilts were confirmed negative for *M. hyopneumoniae*. Throughout the transmission experiment, all naive gilts remained *M. hyopneumoniae* negative by PCR, regardless of treatment group. Therefore, the two CT gilts and one CTV gilt that were *M. hyopneumoniae* negative and all CC gilts were euthanized at this time point.

On postmortem examination, macroscopic and microscopic lung lesions were absent in all CC, CT, and CTV gilts. All bronchial swabs obtained from the CC gilts were positive for *M. hyopneumoniae*, with a mean Ct value of 29.6±0.9 (data not shown). Except for one CT gilt (28.7 Ct value), bronchial swabs collected from the euthanized CT and CTV gilts were negative for *M. hyopneumoniae.* Moreover, bacterial growth was not observed via aerobic culture in lung tissue of all gilts.

4.4.4 Persistence of M. hyopneumoniae

Two CT and two CTV gilts were sampled for an additional 56 days as they remained *M. hyopneumoniae* positive by PCR at 99 dpi but did not transmit the pathogen to their corresponding naïve gilt. From 99 to 155 dpi, inconsistent detection of *M. hyopneumoniae* in tracheal secretions was observed in one CT and one CTV gilt (Table 4.3). *Mycoplasma hyopneumoniae* was detected in one CT and one CTV gilt until 127 and 155 dpi, respectively. At the end of the study, only two gilts (CT and CTV) remained *M. hyopneumoniae* positive based on parallel interpretation of PCR results. Macroscopic lung lesions (4 and 7%) were observed in one CT gilt and one CTV gilt, respectively, in which microscopic lesions suggestive of *M. hyopneumoniae* infection were only identified in the CT gilt (grade=5; data not shown). Bacterial growth was not observed from lung tissue postmortem.

4.4.5 Transmission parameters

Numerical differences in the transmission parameters during the acute infection phase were calculated based on treatment group (Table 4.4). The mean rate at which a naive gilt became infected per day was highest when housed with AC gilts (λ=0.059; 95% CI: 0.008,0.09), followed by ATV gilts (λ=0.021; 95% CI: 0.001, 0.094) and then AT gilts (λ=2.318E-11; 95% CI: NA, 0.034). Moreover, the mean incidence rate for AC gilts was 0.038, implying that during a 14-day period, there were 0.038 new *M. hyopneumoniae* infections per day at risk. In conditions where gilts were vaccinated and/or treated, the incidence rate numerically decreased (Table 4.4). During the chronic infection phase, *M. hyopneumoniae* transmission was not evident as all naive gilts remained negative.

4.5 Discussion

In this study, changes in *M. hyopneumoniae* detection and infectious potential were assessed after the administration of antibiotics and vaccination during the acute and chronic phases of infection. Therapeutic treatment of acute *M. hyopneumoniae* infection with tulathromycin resulted in a numerical reduction in relative bacterial load within the lower respiratory tract of infected gilts compared to their untreated counterparts. Macroscopic lung lesions were also numerically lower in treated, acutely infected gilts compared to non-treated gilts. Moreover, the rate at which naïve gilts became infected was numerically reduced when co-housed with infected gilts that received antibiotic treatment during the acute infection phase, as compared to infected gilts receiving no treatment. During the chronic infection phase, *M. hyopneumoniae* clearance was detected in over a quarter of the treated, infected gilts at 99 dpi. Inconsistent detection of *M.*

hyopneumoniae by PCR was also apparent for the treated, infected gilts, especially as the infection duration progressed. Differences in transmission rates based on treatment group could not be assessed during the chronic infection phase, as all naïve gilts remained *M. hyopneumoniae* negative, including those in contact with non-treated, infected gilts.

Antibiotic treatment for *M. hyopneumoniae* infection has been well-described to decrease bacterial load in the respiratory tract and to decrease clinical severity, lung lesions, and mortality associated with the disease and secondary respiratory bacterial infections (reviewed by Maes et al., 2020b). In this study, a numerical reduction in bacterial load was observed when two tulathromycin treatments were administered during the acute or chronic infection phase. For acutely infected gilts that were treated and vaccinated, the effect of tulathromycin on *M. hyopneumoniae* bacterial load was observed to a lesser degree than in gilts that were only treated. One potential reason for a minimal reduction in bacterial load in treated and vaccinated, infected gilts was the vaccination timing, as this has been thought to be influential towards altering *M. hyopneumoniae* infection (Maes et al., 2008). Since gilts were vaccinated on the same day the inoculation took place, the vaccination timing likely did not provide a strong immune response to maximize the beneficial effects of the intervention with respect to the initial onset of infection. Treatment with tulathromycin also resulted in up to a 17.6% numerical reduction of macroscopic lung lesions during the acute infection phase, thus supporting previous results described by McKelvie et al. (2005) who showed a 8.4% reduction in lung lesions for tulathromycin-treated pigs. Beneficial effects of tulathromycin on health parameters may result as a direct effect of the antibiotics' bacteriostatic properties to inhibit *M. hyopneumoniae* replication, as well as potential indirect elicited anti-

inflammatory and immunomodulatory effects (Duquette et al., 2015). Compared to other antibiotic classes, studies have shown macrolides to induce neutrophil apoptosis and efferocytosis and inhibit several proinflammatory mediators using *in vitro* and *in vivo* models with PRRSv and *Actinobacillus pleuropneumoniae* (Duquette et al., 2015; Moges et al., 2018; Desmonts de Lamache et al., 2019). Anti-inflammatory and immunomodulatory effects of tulathromycin are perceived to be important for *M. hyopneumoniae* disease modulation, as lung lesion development is mainly attributed to the host immune response, resulting in an influx of proinflammatory cytokines and innate immune cells to infected tissue (Kuhnert and Jores, 2020). Nevertheless, additional research is needed to understand the anti-inflammatory and immunomodulatory effects of tulathromycin on *M. hyopneumoniae* infection and disease.

Traditionally, antibiotic treatment for *M. hyopneumoniae* infection has been thought to decrease the transmission of this pathogen by hindering the bacterium's ability to replicate in the respiratory tract, thus reducing the bacterial load that can be transmitted between individual pigs. However, the causal relationship between antibiotics and their effect on *M. hyopneumoniae* transmission has not been previously evaluated. Insight on whether antibiotics can alter pathogen transmission, and to what extent, is essential to optimize control and elimination efforts in the field. To best evaluate this relationship, a 1:1 *in vivo* transmission model was conducted. In the present study, antibiotic treatment altered *M. hyopneumoniae* transmission during the acute infection phase, implying that new infections can still occur but at a lessened rate. Differences in *M. hyopneumoniae* transmission were not observed across the treatment groups due to wide and overlapping confidence intervals for the transmission parameters, which was likely attributed to the

small sample size. Moreover, differences in *M. hyopneumoniae* transmission during the chronic infection period could not be evaluated, as all naïve gilts remained negative until the end of the transmission experiment. Considering the long duration of shedding of *M. hyopneumoniae*, which can reach up to 214 days (Pieters et al., 2009), transmission parameters have been described to be slow and have only been estimated during the acute phase of infection (Meyns et al., 2004; Villarreal et al., 2011a; Betlach et al., 2020). Therefore, the lack of transmission evidenced during the chronic phase was likely the result of the short contact period (i.e., 2 weeks) and the potentially lower risk of pathogen spread, especially due to a potentially lower amount of *M. hyopneumoniae* that may be shed during this time of the infection. Additional research encompassing longer exposure periods and larger populations are warranted to confirm the findings of this study.

In general, bacterial persistence post-treatment may result from biphasic killing kinetics or bacterial population heterogeneity in response to antibiotics due to the presence of 'persister cells', which are described as non- or slow- dividing cells that are not antibiotic-resistant but display a higher threshold of antibiotic susceptibility compared to other cells (Balban et al., 2019). Bacterial persistence can also be facilitated by several factors, including intrinsic or acquired antibiotic resistance, impairment of innate immune defenses, and pathogenic mechanisms to promote immune system evasion or tolerance (Balaban et al., 2019). For *M. hyopneumoniae,* the presence of persistent cells has been hypothesized, as pathogen detection by PCR, clinical signs, and/or lesions has been shown after the administration of antibiotics (Le Carrou et al., 2006; reviewed by Maes et al., 2020b). In this study, persistence of *M. hyopneumoniae* genetic material detection by PCR was shown by the lack of clearance for most infected gilts treated during the chronic

infection phase. Moreover, *M. hyopneumoniae* genetic material was detected by PCR up to approximately three months after receiving antibiotic treatment. However, pathogen transmission from persistently infected pigs was not shown. Drivers for *M. hyopneumoniae* persistence continue to remain unknown and are difficult to assess with the existing diagnostic techniques available. Studies have described antibiotic resistance (reviewed by Maes et al., 2020b), as well as potential biofilm formation for *M. hyopneumoniae* (Tassew, et al., 2017; Raymond et al., 2018). Moreover, the long generation time for *M. hyopneumoniae* (i.e., 6-10 hours; Stemke and Robertson, 1990; Meyns et al., 2007) may contribute to bacterial persistence. Nevertheless, additional research is needed to understand the mechanisms that aid *M. hyopneumoniae* persistence, as well as to develop alternative methods to assess pathogen viability.

During the chronic infection phase, it is important to mention that false negatives results were identified, as samples that resulted suspect or negative for *M. hyopneumoniae* detection by PCR originated from pigs deemed positive at later samplings. Such variation may be explained by intrinsic lack of consistency with sampling and low bacterial loads in the tracheal secretions. The implications of these findings are important to consider when interpreting diagnostic results in the field, especially when surveillance testing is conducted at the end of an elimination program, in which tulathromycin is often administered.

4.6 Conclusions

Based on the conditions of this study, the administration of tulathromycin modified *M. hyopneumoniae* infectious potential by numerically reducing the relative bacterial load and the rate of new infections for treated gilts compared to those non-

treated during the acute phase of infection. Inconsistent detection, as well as persistence of *M. hyopneumoniae* detection by PCR post-treatment was evident during the chronic phase of infection. Further research focused on the effect of antibiotic treatment on *M. hyopneumoniae* viability and transmission and the development of alternative methods to assess pathogen viability is warranted to aid in control and elimination efforts.

Table 4.1 *In vivo Mycoplasma hyopneumoniae* detection by PCR and seroconversion in infected gilts and corresponding naïve gilts

	Infected gilts (% positive; evaluated/total) (Mean value ±SD)			Corresponding naive gilts (% positive; evaluated/total) (Mean value ±SD)		
***Mycoplasma hyopneumoniae* detection by PCR**						
	Treatment group			Treatment group		
Acute	**AC**	**AT**	**ATV**	**AC**	**AT**	**ATV**
0 dpc	100 (4/4)	100 (4/4)	100 (4/4)	0 (0/4)	0 (0/4)	0 (0/4)
	21.3 ±1.3	24.8 ±1.7	22.9 ±1.9	---	---	---
11 dpc	100 (4/4)	100 (4/4)	100 (4/4)	25 (1/4)	0 (0/4)	25 (1/4)
	21.6 ±2.1	23.8 ±1.2	22.1 ±2.5	29.7	---	33.9
14 dpc	100 (4/4)	100 (4/4)	100 (4/4)	50 (2/4)	0 (0/4)	0 (0/4)
	22.1 ±3.0	23.8 ±3.4	20.5 ±2.7	33.65 ±2.8	---	---
Chronic	**CC**	**CT**	**CTV**	**CC**	**CT**	**CTV**
0 dpc	100 (3/3)	75 (3/4)	67 (2/3)	0 (0/3)	0 (0/4)	0 (0/3)
	32.1 ±3.3	28.5 ±1.6	25.2 ±0.7	---	---	---
11 dpc	67 (2/3)	50 (2/4)	67 (2/3)	0 (0/3)	0 (0/4)	0 (0/3)
	29.5 ±2.6	30.3 ±0.2	34.0 ±1.1	---	---	---
14 dpc	100 (3/3)	50 (2/4)	67 (2/3)	0 (0/3)	0 (0/4)	0 (0/3)
	29.2 ±4.4	32.2 ±0.7	30.4 ±5.2	---	---	---
Seroconversion to *Mycoplasma hyopneumoniae* by ELISA						
	Treatment group			Treatment group		
Acute	**AC**	**AT**	**ATV**	**AC**	**AT**	**ATV**
0 dpc	75 (3/4)	25 (1/4)	100 (4/4)	0 (0/4)	0 (0/4)	0 (0/4)
	0.9 ±0.2	0.9 ±0.0	1.2 ±0.1	---	---	---
14 dpc	100 (4/4)	100 (4/4)	100 (4/4)	0 (0/4)	0 (0/4)	0 (0/4)
	1.2 ±0.5	0.7 ±0.3	1.5 ±0.2	---	---	---
Chronic	**CC**	**CT**	**CTV**	**CC**	**CT**	**CTV**
0 dpc	100 (3/3)	100 (4/4)	100 (3/3)	0 (0/3)	0 (0/4)	0 (0/3)
	1.7 ±0.3	1.0 ±0.3	1.5 ±0.2	---	---	---
14 dpc	100 (3/3)	100 (4/4)	100 (3/3)	0 (0/3)	0 (0/4)	0 (0/3)
	1.7 ±2.5	0.9 ±2.2	1.4 ±2.8	---	---	---

Treatment group: AC (acutely infected, control); AT (acutely infected and treated); ATV (acutely infected, treated and vaccinated); CC (chronically infected, control); CT (chronically infected and treated); CTV (chronically infected, treated and vaccinated). dpc=days post-contact. For *M. hyopneumoniae* detection and seroconversion, values represent mean Ct and S/P values by PCR and Idexx ELISA, respectively. SD=standard deviation.

Table 4.2 Postmortem *Mycoplasma hyopneumoniae* detection by PCR, lung lesion severity (macroscopic and microscopic), and bacterial culture in acutely, infected gilts and corresponding naïve gilts

	Infected gilts (%; evaluated/total)			**Corresponding naïve gilts** (%; evaluated/total)		
	Treatment group			**Treatment group**		
Macroscopic lung lesion score (%)	**AC**	**AT**	**ATV**	**AC**	**AT**	**ATV**
0	---	---	---	100 (4/4)	50 (2/4)	25 (1/4)
1-5	---	50 (2/4)	25 (1/4)	---	25 (1/4)	75 (3/4)
6-10	---	---	25 (1/4)	---	25 (1/4)	---
11-15	---	---	---	---	---	---
15-20	---	25 (1/4)	50 (2/4)	---	---	---
21+	100 (4/4)	25 (1/4)	---	---	---	---
Average	26.0%	8.4%	9.9%	0%	0.8%	1.8%
Histopathologic score (0-5)						
0	---	25 (1/4)	---	75 (3/4)	25 (1/4)	50 (2/4)
1	---	---	33 (1/3)*	25 (1/4)	25 (1/4)	50 (2/4)
2	---	---	---	---	25 (1/4)	---
3	75 (3/4)	---	33 (1/3)*	---	25 (1/4)	---
4	25 (1/4)	75 (3/4)	33 (1/3)*	---	---	---
5	---	---	----	---	---	---
Median	3	4	3	0	1.5	0.5
Bronchial swab by PCR (Mean Ct value ±SD)	100 (4/4) 19.4 ±0.8	100 (4/4) 21.4 ±1.7	100 (4/4) 21.3 ±0.5	0 (0/4)	0 (0/4)	0 (0/4)

Aerobic culture growth	*G. parasuis* (1/4)	*G. parasuis* (1/4)	---	---	*G. parasuis* (1/4)	---

Treatment group: AC (acutely infected, control); AT (acutely infected and treated); ATV (acutely infected, treated and vaccinated). *=histopathologic score was not evaluated in one of the four ATV pigs due to loss of tissue sample. SD=standard deviation. *G. parasuis*= *Glaesserella parasuis.*

Table 4.3 Persistence of *Mycoplasma hyopneumoniae* detection by PCR during the chronic infection phase

	Days post-inoculation					
Treatment group	99	113	127	144	155	155*
CT	**31.44**	**26.58**	**29.1**	39.56	39.59	---
CT	**32.91**	39.28	**34.5**	---	---	**28.72**
CTV	**35.59**	39.54	---	**34.97**	---	---
CTV	**25.18**	**30.23**	**34.9**	**32.83**	**34.01**	---

Treatment group: CT (chronically infected and treated); CTV (chronically infected, treated and vaccinated). For *M. hyopneumoniae* detection, values represent mean Ct by PCR. Positive PCR results are bolded in black. Dashed lines represent a negative PCR result. *=detection in bronchial swabs

Table 4.4 *Mycoplasma hyopneumoniae* transmission parameters during the acute infection phase

Treatment group	Force of infection (95% CI)	Incidence rate (95% CI)
AC	0.059 (0.008, 0.090)	0.038 (0.004, 0.136)
AT	2.318E-11 (NA, 0.034)	0.000 (0.000, 0.066)
ATV	0.021 (0.001, 0.094)	0.019 (0.000, 0.105)

Treatment group: AC (acutely infected, control); AT (acute infected and treated); ATV (acutely infected, treated and vaccinated). CI=confidence interval. NA=not available

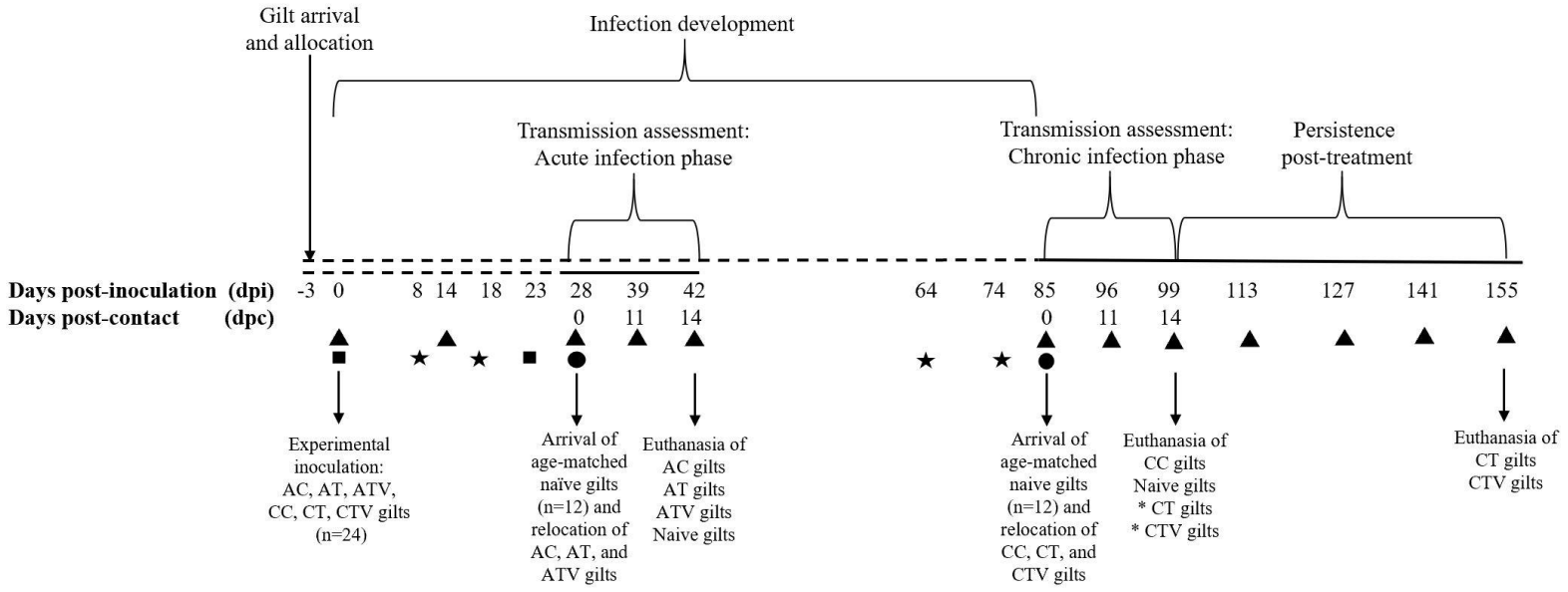

Figure 4.1 Study experimental design

Treatment group: AC (acute, control); AT (acute, treated); ATV (acute, treated and vaccinated); CC (chronic, control); CT (chronic, treated); CTV (chronic, treated and vaccinated). Dashed lines correspond to infection development for the acute or chronic phase of infection, whereas solid lines indicate infection and transmission assessment for each phase. Black squares represent when *M. hyopneumoniae* bacterin was administered to ATV and CTV gilts (n=8). Black stars represent when tulathromycin was administered to AT, ATV, CT, and CTV gilts (n=16). Black circles indicate when naïve gilts were initially placed in contact with infected gilts. Black triangles represent sampling events. Asterisks indicate that euthanasia of CT and CTV gilts was dependent on PCR results.

Chapter 5: Characterization of circulating *Mycoplasma hyopneumoniae* variants in the Midwestern United States using Multiple-Locus Variable number tandem repeat Analysis and P146 gene sequencing

5.1 Summary

In the United States, Multiple-Locus Variable number tandem repeat Analysis (MLVA) and complete P146 gene sequence are commonly employed to molecularly characterize *Mycoplasma hyopneumoniae* from clinical samples. However, the comparison of MLVA and P146 sequencing and interpretation of assay results remains unclear. Therefore, the aim of this study was to characterize and compare *M. hyopneumoniae* variants in the Midwestern United States using MLVA and P146 gene sequencing. A total of 160 samples were analyzed for this investigation. Molecular techniques were compared for assay sensitivity, discriminatory power, and congruence. Epidemiological relationships in clustering of *M. hyopneumoniae* variants were evaluated by employing Principal Coordinate Analysis. Fair agreement (κ=0.34) in assay outcome was calculated between the two techniques. Ability to obtain a VNTR type or a P146 sequence was dependent upon the relative bacterial load (i.e., Ct value) in the sample. The Simpson's diversity index was higher for MLVA (D=0.899) than for P146 sequencing (D=0.844). High congruence for the number of tandem repeats detected in the polyserine region of P146 was also obtained. Similar epidemiological inferences were generated from the two assays, as production flow explained most of the variation in the clustering of VNTR types and P146 sequences. Findings from this study highlighted differences in assay sensitivity and discriminatory power between the two molecular techniques. Nevertheless, both techniques showed a wide genetic diversity among *M. hyopneumoniae* variants, and similar epidemiological inferences were generated. Further research utilizing whole-genome sequencing should be conducted to evaluate the

presence of other variable areas within the genome that are unrepresented by MLVA and P146 sequencing.

5.2 Introduction

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is the etiologic agent of enzootic pneumonia (Maré and Switzer, 1965; Goodwin et al., 1965), a chronic bacterial respiratory disease in swine known to result in major health and economic concerns. Enzootic pneumonia is characterized by a dry cough, reduced gain, and poor feed efficiency (Pointon et al., 1985; Straw et al., 1989). As a main contributor to the Porcine Respiratory Disease Complex, *M. hyopneumoniae* infections predispose pigs to secondary bacterial and viral pathogens, which further exacerbates respiratory disease challenges (Pieters and Maes, 2019).

Intra-herd transmission of *M. hyopneumoniae* primarily occurs through direct contact between infected and susceptible pigs, including dam-to-piglet interaction. In sow herds, circulation of *M. hyopneumoniae* occurs among infected dams and incoming susceptible gilts, which is thought to maintain ongoing pathogen transmission due to the constant introduction of gilts year-round (Maes et al., 2018). Piglets are considered a secondary population that aid in pathogen transmission, as they are born free of the microorganism, but are later colonized from shedding dams during the lactation period (Calsamiglia and Pijoan, 2000a). Due to the long shedding duration of *M. hyopneumoniae* (Pieters et al., 2009), which can last up to 7 months, infected sow herds are considered the reservoir for *M. hyopneumoniae* for downstream grower-finisher herds (Fano et al., 2005; Maes et al., 2018). Nevertheless, inter-herd transmission of *M.*

hyopneumoniae via aerosol has been suspected to occur over long distances (Goodwin, 1985).

Pathogen origin can be inferred with an understanding of genetic relatedness between variants detected within and/or among herds, different geographic locations and/or time periods (Wang et al., 2015). As the cornerstone of molecular epidemiology, molecular characterization techniques should be accurate, feasible, reproducible, and with high discriminatory power (Wang et al., 2015). For *M. hyopneumoniae,* molecular characterization techniques are essential to link pathogen transmission between herds and support control and elimination efforts. Several *M. hyopneumoniae* molecular characterization techniques based on nucleic acid amplification, genome typing or sequencing have been described (reviewed by Betlach et al., 2019). In the United States, Multiple-Locus Variable number tandem repeat Analysis (MLVA) and sequencing of the complete P146 gene are two commonly employed techniques to characterize *M. hyopneumoniae* directly from clinical field samples or isolates. Given that *Mycoplasma spp*. genomes have numerous repetitive regions that undergo phase variation or recombination, MLVA typing (Vranckx et al., 2011) and P146 gene sequencing have been developed to target genomic areas with known highly variable regions and established bacterial adhesion functionality. Multiple MLVA techniques have been described using different loci (reviewed by Betlach et al., 2019). In the United States, *M. hyopneumoniae* MLVA typing has been standardized using two loci (Dos Santos et al., 2015), namely P97 an adhesin protein, and P146, a surface protein with paralogous nature with P97 (Minion et al., 2004). Moreover, the gene that encodes for P146, has been

primarily sequenced as it contains many unique features, including two putative coiled-coil domains and three variable repeat regions (Bogema et al., 2012).

During recent years, there has been a pressing need to utilize *M. hyopneumoniae* molecular characterization techniques in the field for epidemiological investigations. However, there is limited information on the interpretation and comparison of MLVA and complete gene sequencing of P146, along with the epidemiological insight gained from the genomic analyses. Therefore, the aim of this study was to characterize and to compare *M. hyopneumoniae* variants in the Midwestern United States using MLVA and P146 gene sequencing.

5.3 Materials and methods

5.3.1 Clinical field samples

A total of 160 clinical samples were conveniently collected between 2013 and 2018 from 84 unique swine herds in five production systems that were predominately located in the Midwestern region of the United States. Samples, including laryngeal and bronchial swabs, and tracheal secretions, were submitted to the University of Minnesota Veterinary Diagnostic Laboratory (UMN-VDL) for routine diagnostic and/or surveillance purposes. Samples were processed and the genetic material was extracted and tested for *M. hyopneumoniae* real-time PCR (Strait et al., 2008), according to standardized protocols at the diagnostic laboratory. Samples were representative of *M. hyopneumoniae* cases within the Midwestern US swine industry as sampled herds were located in states that accounted for ~61% of the hog production inventory in the Midwest (Livestock Slaughter Annual Summary, USDA, NASS, 2018). Moreover, epidemiological information, including date, geographical location (i.e., state), production system, flow,

and stage were collected. In this study, a production flow is defined as a group of herds that share a common pig source and health status and is comprised of herds with different production stages (e.g., sow herd, nursery, finishing).

5.3.2 Multiple-Locus Variable number tandem repeat Analysis

The MLVA assay was attempted for the 160 samples, as described by Dos Santos et al. (2015). In addition, the *M. hyopneumoniae* ATCC 25095 reference strain was included to serve as a positive control. Briefly, the MLVA assay detected and quantified the number of tandem repeats for two loci, namely repeat region 1 (RR1) of P97 (*Mhp* 138) and RR3 of P146 (*Mhp* 684). Genetic material for the two loci was amplified by performing a touchdown multiplex PCR modified from Vranckx et al. (2011). For the PCR, 0.2 μM of dye-labeled primers, 1X Qiagen Multiplex PCR Master Mix (Qiagen, CA, USA), and 2μL of DNA were combined for a final volume of 25 μL. PCR products were then analyzed by gel electrophoresis to confirm product amplification and later submitted for capillary electrophoresis. Data files from the capillary electrophoresis were imported into a bioinformatics analytic software (BioNumerics, version 7.1, Applied Maths, Austin, TX, USA), and computational parameters described by Dos Santos et al. (2015) were employed to assess fragment size and determine the number of tandem repeats for each locus. A minimum spanning tree was constructed to illustrate clustering based on categorical values of Variable Number Tandem Repeat (VNTR) type. Samples with an incomplete or partial VNTR type were removed from the clustering analysis.

5.3.3 Complete gene sequencing of P146

P146 sequencing was conducted at the UMN-VDL using an *in-house* procedure that employed Sanger sequencing. Sequences from multiple Sanger runs were assembled and aligned using ClustalW to create a consensus sequence. Sequence analysis was performed using Geneious 10.2.3. For the analysis, sequences from five publicly available *M. hyopneumoniae* reference strains (i.e., strain 232 from United States – Accession: AE017332.1; J strain from Brazil –Accession: AE017243.1; 7448 strain from Brazil –Accession:AE017244.1; 168 strain from China –Accession: CP002274.1; F7.2C strain from Belgium –Accession: DQ088147.1; and BQ14 strain from France – Accession: AF279908) were included. Partial sequences were not included in the analysis. Sequences were aligned using MUSCLE algorithm with default settings, followed by translation of nucleotides to amino acids. A maximum likelihood phylogenetic tree was constructed for protein sequence alignments using RAxML v7.3.0 (Stamatakis, 2006) based on 1,000 bootstrap replicates and default settings for JTT matrix-model. Microreact (Argimón et al., 2016) was employed to illustrate the phylogenetic tree.

5.3.4 Comparison of molecular characterization techniques

5.3.4.1 Assay performance

Assay sensitivity was descriptively compared for MLVA and P146 sequencing. Logistic regression models were used to estimate the odds of obtaining a VNTR type or P146 sequence based on *M. hyopneumoniae* PCR Ct values. Agreement in assay outcome between the two molecular techniques was measured using Cohen's kappa index. Level of agreement based on Kappa (κ) value was interpreted as follows: <0.00=poor; 0.00-0.20=slight; 0.20-0.40=fair; 0.41-0.60=moderate; 0.61-0.80=substantial; 0.81-

1.00=almost perfect (Landis and Koch, 1977). Data analyses were performed using R 3.6.1 (R Core Team, 2019) and results with a *p*-value <0.05 were deemed significant.

5.3.4.2 Discriminatory power and assay congruence for RR3 P146 serine repeats

Discriminatory power was estimated by calculating Simpson's index of diversity for MLVA using each targeted loci and their combination (i.e., VNTR type), as well as for P146 sequencing, with the Hunter-Gaston diversity index (Hunter and Gaston, 1988). The overall congruency and interchangeability between the two molecular techniques for the identification of serine repeats in RR3 P146 were estimated and compared by calculating the adjusted Rand (Hubert and Arabie, 1985) and adjusted Wallace coefficients (Pinto et al., 2008) using Comparing Partitions (Comparing Partitions, http://www.comparingpartitions.info/index.php?link=Home, accessed March 2, 2021). Adjusted Rand provides a measure of the overall agreement between two methods, whereas, the adjusted Wallace test provides information regarding directionality of agreement (Severiano et al., 2011). Therefore, inferences related to the probability that samples are classified together with a particular method based on their previous classification with a different method can be obtained.

5.3.4.3 Epidemiological relationship

Clustering of *M. hyopneumoniae* variants based on several epidemiological variables, including year, geographical location, and production flow, were explored by employing Principal Coordinates Analysis (PCoA) plots. For MLVA, PCoA plots were generated by Jaccard distance using phyloseq package (McMurdie and Holmes, 2013) in R (R Core Team). Similarly, PCoA plots were created for P146 sequences using the

percent difference in amino acids as a distance matrix. Clustering of sequences based on serine repeats was also evaluated. Cluster analysis was conducted using adonis and betadisper tests from the vegan package (Oksanen et al., 2020) in R.

5.4 Results

5.4.1 Assay performance

The sensitivity of MLVA and P146 sequencing was 88.1% (141/160) and 78.7% (126/160), respectively, in which both a VNTR type and a P146 sequence were obtained for 75% (120/160) of the samples. Partial results were detected for 3.1% (5/160) and 10% (16/160) of the samples for MLVA and P146 sequencing, respectively. For MLVA, a VNTR type was not identified for 8.7% (14/160) of the samples. Similarly, a P146 sequence was not obtained for 11.2% (18/160) of the samples. There was fair agreement (κ=0.34; 95% CI: 0.13-0.55) for assay outcome between the two molecular techniques. A significant relationship between Ct value and the ability to obtain a VNTR type or a P146 sequence was observed (Figure 5.1). Since linearity assumption was not met, Ct values were divided into four groups: C1 (Ct values ≤20), C2 (Ct values 21-26), C3 (Ct values 27-32), and C4 (Ct values ≥33). The estimated odds of obtaining a VNTR type was 67.4% and 97.3% lower for samples belonging to C3 (OR: 0.326; *p*-value=0.31) and C4 (OR: 0.028; *p*-value<0.01) compared to C1 samples, respectively. Regarding P146 sequencing, the estimated odds of obtaining a sequence was 85.2% lower for C3 samples than C1 samples (OR: 0.148; *p*-value=0.07). Odds for obtaining a sequence with C4 samples was similar to those seen for VNTR type.

5.4.2 Characterization of M. hyopneumoniae *variants based on assay*

Mycoplasma hyopneumoniae genetic diversity was evaluated in 120 samples using MLVA typing and P146 sequencing. Percentage of samples based on year of collection is as follows: 2013 (15.8%), 2014 (5.8%), 2015 (34.2%), 2016 (36.7%), 2017 (4.2%), and 2018 (3.3%). The samples were collected from 71 unique herds of various production stages, including finishing (37.5%), gilt developing units (GDU; 26.7%), sow (23.3%), nursery (5.0%), and wean-to-finish sites (2.5%), with 5.0% of samples of unknown stage origin. Moreover, production flow information was obtained for most of the sampled herds, accounting for the evaluation of *M. hyopneumoniae* genetic diversity across 18 production flows.

5.4.2.1 Multiple-Locus Variable number tandem repeat Analysis

Thirty-four VNTR types were identified using MLVA (Figure 5.2). The most frequent VNTR types were: 13-17 (22.5%), 16-24 (20.0%), 19-19 (7.5%), and 3-15 (6.7%). In this study, the number of tandem repeats for P97-RR1 and P146-RR3 ranged from two to 19, and from 12 to 27, respectively. Across the five production systems, four common VNTR types (i.e., 13-17, 16-18, 16-26, and 2-19) were identified, which accounted for 34% (41/120) of the samples collected. Within a single production flow, up to five VNTR types were identified (data not shown). At the herd-level, a single VNTR type was detected in a majority of herds (90.1%). For 9.8% (7/71) of the herds, two or three VNTR types were detected during one sampling event, in which VNTR types differed by no more than two tandem repeats in either one or both loci (data not shown).

5.4.2.2 P146 sequencing

Mean percentage amino acid pairwise genetic distance for field sequences (n=120) was 2.9% (Table 5.1). Field sequences were more genetically similar to *M. hyopneumoniae* J strain from Brazil and strain 232 from the United States than strains 7448, BQ14, 168, and F7.2C, which originated from Brazil, France, China, and Belgium, respectively (Table 5.1; Figure 5.3). Clustering of sequences based on serine repeat number was depicted in a phylogenetic tree (Figure 3) and supported through the employment of PCoA plots. In the Midwestern United States, serine repeat number accounted for 65.5% of the variation between the P146 sequences (R^2=0.655; *p*-value<0.01). Sequence variation was less associated with serine repeat number when sequences of greater genetic diversity (i.e., *M. hyopneumoniae* reference strains) were included (R^2=0.231; *p*-value<0.01).

5.4.2.3 Discriminatory power

The Simpson's diversity index for each loci and their combination for MLVA, as well as for P146 sequencing, is provided in Table 5.2. A significant difference in discriminatory power was calculated between MLVA (*D*=0.899) and P146 sequencing (*D*=0.844; *p*-value<0.01).

5.4.2.4 Assay congruence for P146-RR3 serine repeats

The number of serine repeats for P146-RR3 was compared between the two molecular techniques, resulting in high congruency, overall (ARI=0.910; CI: 0.835-9.986; Table 5.2). Of the 120 samples, 6.6% (8/120) showed numerical differences in serine repeat count, ranging between 1 and 5 repeats. Directional agreement in serine repeats between MLVA and P146 sequence was evaluated by calculating the adjusted

Wallace coefficients. The value of $AW_{\text{MLVA P146 loci}\rightarrow\text{Sequencing}}$ of 0.942 (CI: 0.890-0.994) was not significantly higher than the agreement measured by $AW_{\text{Sequencing}\rightarrow\text{MLVA P146 loci}}$ of 0.880 (CI: 0.794-0.965; p=0.15; Table 5.2), indicating bidirectional correspondence.

5.4.3 Epidemiological relationship

Clustering of *M. hyopneumoniae* variants based on several epidemiological variables was explored by employing Principal Coordinates Analysis (PCoA) plots (Figure 5.4). Table 5.3 depicts variance in VNTR types and genetic distance for P146 sequences based on production flow and stage, state, and year. Dissimilarities in VNTR type and P146 sequences were significantly associated with pig production flow, in which flow accounted for the highest amount of variation (Table 5.3). Variation in VNTR type and P146 sequences was minimally explained by production stage, state, and year.

5.5 Discussion

Mycoplasma hyopneumoniae variants in the Midwestern United States were characterized and compared using two commonly employed molecular techniques, MLVA and P146 sequencing. Molecular techniques were compared based on assay sensitivity, discriminatory power, and congruency. The ability to obtain a result varied between the two molecular techniques. However, assay outcome was dependent upon the relative bacterial load (i.e., Ct value) in the clinical sample, regardless of technique. A fair agreement in assay outcome was calculated, as opposed to a moderate or almost perfect agreement, which could have resulted from the higher number of partial or incomplete results obtained for P146 sequencing compared to MLVA. In this study, the molecular techniques were conducted in a parallel manner using the same genetic

material, thus reducing the presence of confounding variables. Nevertheless, variation could have resulted from the intrinsic differences in techniques, along with the corresponding primers utilized, or personnel performing the assay.

Assay sensitivity and discriminatory power are two important features to consider for the employment of a molecular characterization technique, along with cost, feasibility and reproducibility. Molecular techniques with a high discriminatory power aid to further differentiate between variants. Throughout the literature, the characterization of *M. hyopneumoniae* variants and isolates has been shown to vary based on the technique employed. In this study, differences in discriminatory power between MLVA and P146 sequencing were observed. Although the sequencing of the complete P146 gene has not been previously investigated prior to this study, sequencing of the polyserine encoding region of the P146 gene has been conducted and described to have a high discriminatory power (D=0.92; Mayor et al., 2007; Felde et al., 2018). Moreover, several studies have also described a high discriminatory power for MLVA techniques that incorporate the target of P146-RR3 (Vranckx et al.., 2011; Dos Santos et al., 2015; Felde et al., 2018).

High congruency (ARI=0.910) and bidirectional agreement for the number of serine repeats in P146 was generated between the two molecular techniques. Therefore, both assays can be employed to characterize *M. hyopneumoniae* based on the polyserine encoding region of P146. Throughout the P146 gene, there are five highly diverse regions, which include three variable repeat regions and two putative coiled-coil domains. In this study, genetic variation across sequences was less associated with serine repeat number when *M. hyopneumoniae* reference strains were included. Therefore, a comprehensive review of the complete P146 gene is needed, especially with sequences of

high genetic diversity, to further characterize *M. hyopneumoniae* variants and identify regions of the gene that are influential towards genetic variation at the entire gene-level.

To further characterize *M. hyopneumoniae*, whole-genome sequencing should be conducted to evaluate the presence of other variable areas within the genome that are unrepresented by MLVA and P146 sequencing. For other bacterial pathogens, whole-genome sequencing is becoming a widely used technique for research and diagnostic purposes, in which isolates are predominately utilized (He, 2015). For *M. hyopneumoniae*, whole-genome sequencing has been employed for the molecular characterization of seven isolates (reviewed by Jarocki and Djordjevic, 2020). However, the fastidious nature of *M. hyopneumoniae* poses challenges with isolating the bacterium from clinical samples on a routine basis. Therefore, development and validation of procedures to ensure a high integrity and quantity of *M. hyopneumoniae* genetic material from clinical samples, while removing host or other bacterial and viral genetic material, is needed for the application of whole-genome sequencing in the field.

In this study, 34 VNTR types were detected throughout the Midwestern United States, in which the VNTR types were predominately clustered by production flow versus state and year. However, a comparable sample size among Midwestern states and year was not evaluated, thus influencing the number of VNTR types detected and the potential diversity of *M. hyopneumoniae.* To provide further insight towards the molecular epidemiology of *M. hyopneumoniae,* a larger dataset that is composed of several samples collected throughout distinct geographical locations and over time is needed. In doing so, the temporal dynamics of circulating *M. hyopneumoniae* variants may be assessed, which

can help infer bacterial evolution and aid in the differentiation of unique or emerging variants.

5.6 Conclusions

In conclusion, differences in assay sensitivity and discriminatory power between the two molecular techniques were demonstrated, favoring the utilization of MLVA for the characterization of *M. hyopneumoniae* diversity compared to P146 sequencing. However, depending on the question at hand, the construction of phylogenetic trees using P146 sequencing can be utilized to help infer evolutionary relationships among *M. hyopneumoniae* variants. Further research focused on the utilization of whole-genome sequencing to further characterize *M. hyopneumoniae* is warranted, especially to identify regions of the genome that may be of biological significance.

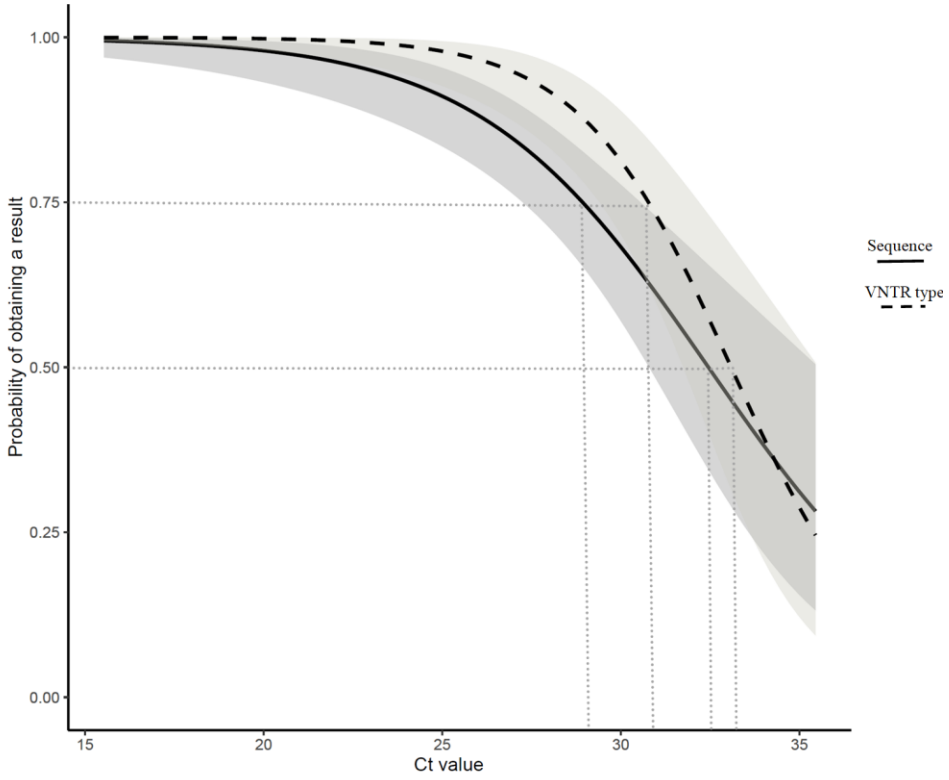

Figure 5.1 Estimated probability of obtaining a complete result using Multiple-Locus Variable number tandem repeat Analysis (VNTR type) or P146 sequencing (sequence) based on *Mycoplasma hyopneumoniae* PCR Ct value

Confidence intervals are depicted by shaded areas: gray (sequence); ivory (VNTR type). Gray dashed lines represent 75% or 50% probability of obtaining either a VNTR type or sequence based on Ct value.

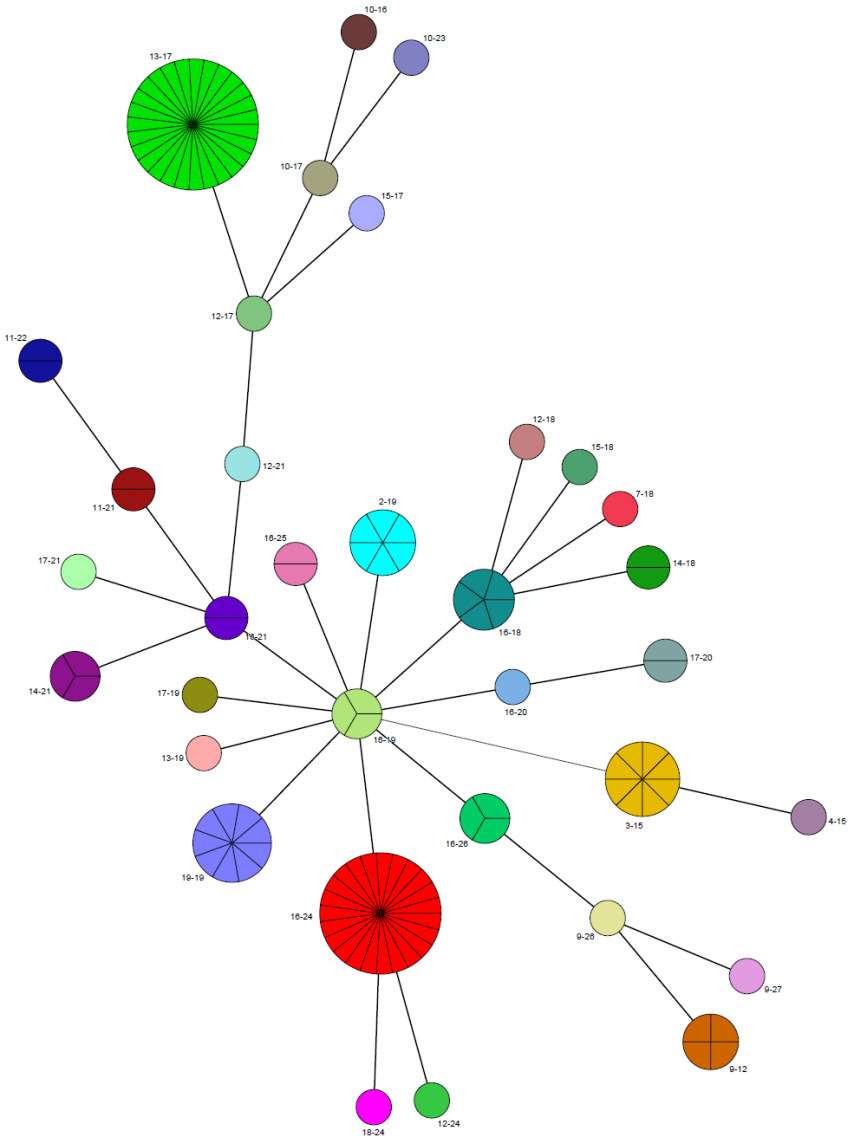

Figure 5.2 Minimum spanning tree for *Mycoplasma hyopneumoniae* Variable Number Tandem Repeat (VNTR) type obtained with Multiple-Locus Variable Number tandem repeat Analysis

Circle size and color corresponds to sample size for each specific VNTR type, respectively. Numbers outside of each circle represent VNTR type for two loci, namely P97 and P146. Lines that connect circles depict relationship between VNTR types.

Table 5.1 Mean genetic distance as percentage difference in amino acids for *Mycoplasma hyopneumoniae* P146 field and reference sequences

	Field sequences	Mhp_232 USA	Mhp_7448 Brazil	Mhp_BQ14 France	Mhp_168 China	Mhp_F7.2C Belgium	Mhp_J Brazil
Field sequences	2.9*						
Mhp_232_USA	3.2	---					
Mhp_7448_Brazil	7.1	6.7	---				
Mhp_BQ14_France	5.6	5.5	8.0	---			
Mhp_168_China	6.5	6.2	7.4	6.9	---		
Mhp_F7.2C_Belgium	7.6	7.1	6.6	8.5	7.1	---	
Mhp_J_Brazil	2.8	3.3	6.2	5.2	6.1	7.0	---

*=Mean genetic distance for all field sequences (n=120). Mhp=*Mycoplasma hyopneumoniae.*

Table 5.2 Comparison of discriminatory power and congruence of assays for *Mycoplasma hyopneumoniae* molecular characterization

Discriminatory power				
	MLVA loci			P146 sequencing
	P97	P146	Combined	
Simpson's index coefficient	0.828 (CI: 0.783-0.873)	0.853 (CI: 0.23-0.883)	0.899[a] (CI: 0.866-0.932)	0.844[b] (CI: 0.814-0.875)

Assay congruency for RR3 P146 serine repeats		
	MLVA P146 loci vs. Sequencing	
Adjusted Rand coefficient	0.910 (CI: 0.835-9.986)	
	$AW_{\text{MLVA P146}\rightarrow\text{Sequencing}}$	$AW_{\text{Sequencing}\rightarrow\text{ MLVA P146}}$
Adjusted Wallace coefficient	0.942[a] (CI: 0.890-0.994)	0.880[a] (CI: 0.794-0.965)

Differences in superscripts indicate statistical significance. *AW*=adjusted Wallace coefficient.

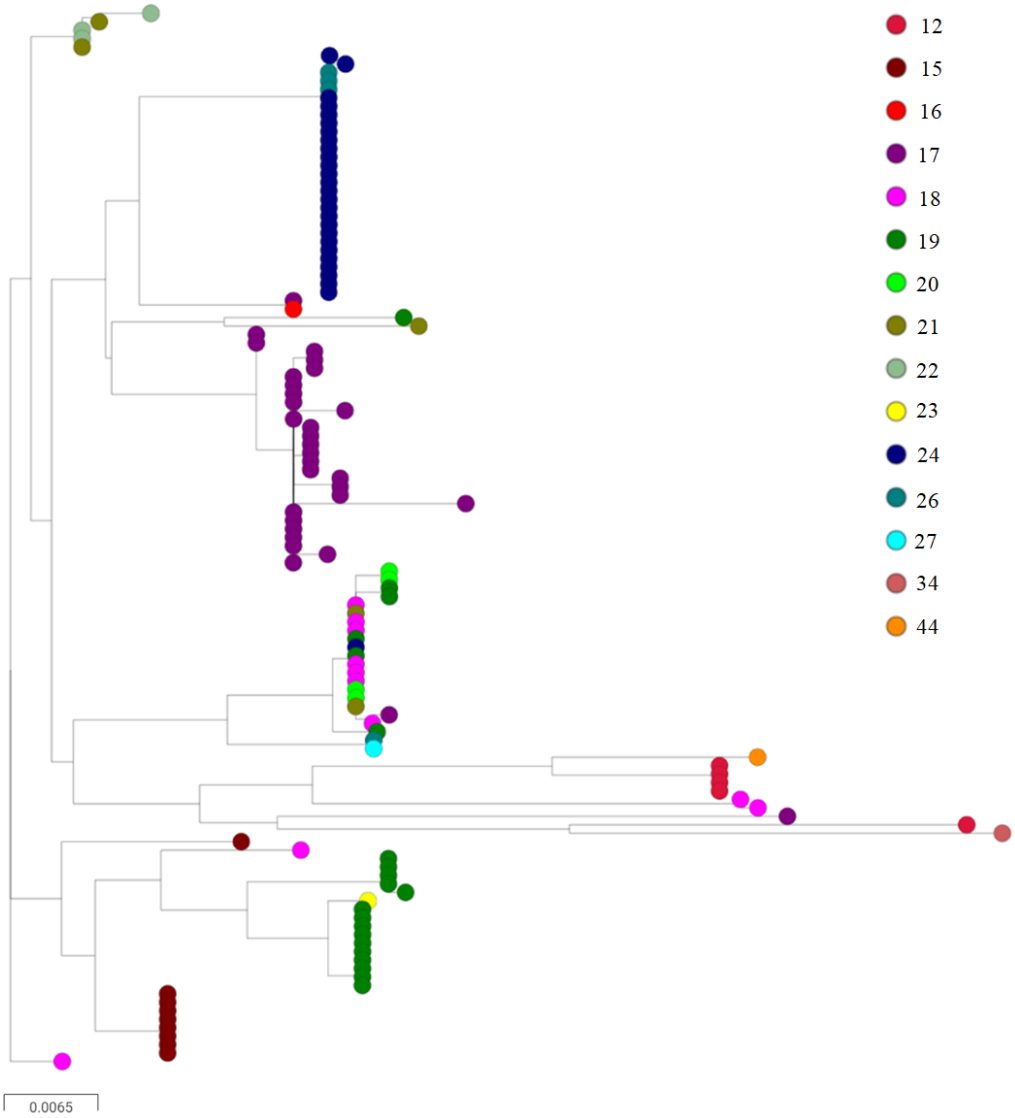

Figure 5.3 Maximum likelihood phylogenetic tree of *Mycoplasma hyopneumoniae* P146 sequences

Colors correspond to the number of serine repeats in P146-RR3. Phylogenetic tree was constructed using RAxML method with default settings for JTT-matrix model. Microreact was employed to illustrate the tree. The bar scale indicates the genetic distance based on amino acids.

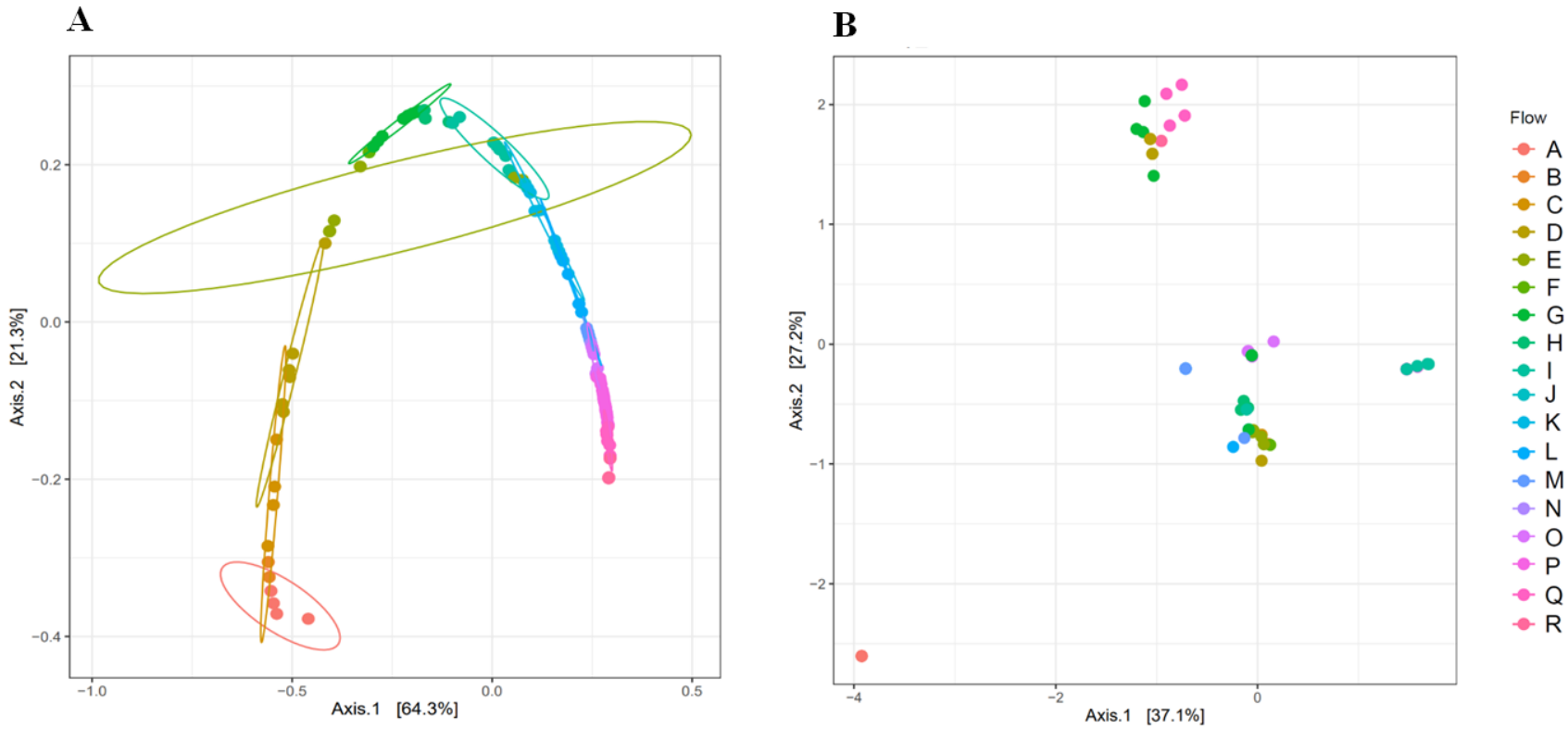

Figure 5.4 Principal Coordinate Analysis (PCoA) plot illustrating clustering of *Mycoplasma hyopneumoniae* Variable Number Tandem Repeat (VNTR) type and P146 sequences by production flow

A. VNTR type. B. Pairwise genetic distance of P146 sequences. Each dot is color-coded based on production flow. Circles in panel A correspond to 95% confidence ellipses.

Table 5.3 Variation in Variable Number Tandem Repeat (VNTR) type and genetic distance of P146 sequences based on epidemiological variables

	VNTR type		P146 sequence	
	R^2	*p*-value	R^2	*p*-value
Production flow	0.931	0.00	0.798	0.00
Production stage	0.050	0.29	0.064	0.09
State	0.126	0.00	0.150	0.00
Year	0.176	0.00	0.261	0.00

Chapter 6: Genetic diversity of *Mycoplasma hyopneumoniae* within swine production flows

Work from this chapter has been published in:

Betlach, A.M., Fano, E., Sponheim, A., Valeris-Chacin, R., Dalquist, L., Singer, R.S., Pieters, M. 2020. *Mycoplasma hyopneumoniae* genetic variability within swine production flows. **Canadian Journal of Veterinary Research**. 84, 310-313.

6.1 Summary

The aim of this study was to assess the genetic variability of *M. hyopneumoniae* within various swine production flows. Four *M. hyopneumoniae* positive production flows, composed of four production stages, were selected for this study. Laryngeal and/or bronchial swabs were collected from each production stage within a flow, for a period of four months up to three years. Multiple-Locus Variable number tandem repeat Analysis (MLVA) was performed to assess the genetic variation of *M. hyopneumoniae* within and across production flows through the identification of Variable Number Tandem Repeat (VNTR) types. A maximum of six *M. hyopneumoniae* VNTR types were identified in a single flow, in which VNTR types appeared to be flow specific. An identical VNTR type was detected across several production stages for up to three years. In this study, minimal *M. hyopneumoniae* genetic variation was evidenced within and across production flows.

6.2 Introduction

Mycoplasma hyopneumoniae (*M. hyopneumoniae*) is the primary agent of enzootic pneumonia (EP) in swine (Maré and Switzer, 1965; Goodwin et al., 1965), a prevalent disease known to have a significantly negative impact in swine health and production worldwide (Pieters and Maes, 2019). Variability in *M. hyopneumoniae* virulence has been presumed due to demonstrated differences in clinical course (Vicca et al., 2002) and disease severity among strains (Vicca et al., 2003). Molecular characterization techniques have been developed and thus described genomic differences among strains (Betlach et al., 2019). Molecular findings have raised interest from veterinarians and researchers towards understanding the influence of *M. hyopneumoniae* variability on disease epidemiology.

Using Multiple-Locus Variable number tandem repeat Analysis (MLVA), studies have shown the presence of multiple *M. hyopneumoniae* Variable Number Tandem Repeat (VNTR) types at a geographic, production flow, herd, and individual pig level (Dos Santos et al., 2015; Rebaque et al., 2018; Pantoja et al., 2016; Michiels et al., 2017). However, the potential source of VNTR type variation has not been fully elucidated. Insight on understanding potential origins and drivers of genetic variation is critical for *M. hyopneumoniae* epidemiological investigations and to help tailor control strategies based on VNTR type(s) and diversity. Therefore, the aim of this study was to assess the genetic variability of *M. hyopneumoniae* within various swine production flows.

6.3 Materials and methods

6.3.1 Flow selection and background

Four production flows (A-D) located in Central United States were selected based on positive status for *M. hyopneumoniae*, history of clinical signs suggestive of infection, and similar management protocols within one production system. In this study, a production flow was defined as a group of sites sharing pig sources and health status, and encompassed at least one herd from each production stage: gilt developing unit (GDU), sow herd, nursery, and finisher. All farms were single sourced by flow. Herds that belonged to each flow were located across different geographical states, except for flow A. The breeding stock source for all flows was presumed *M. hyopneumoniae* negative gilt multipliers that originated within the production system, and their health status was confirmed through routine diagnostics. Upon arrival into the breeding herd, gilts were acclimated through a seeder-to-naïve model by introducing *M. hyopneumoniae* positive culled sows and gilts into flow specific GDUs. Gilt acclimation strategies were utilized in

the GDUs to help to promote *M. hyopneumoniae* stability within the sow herd. Gilts were vaccinated with a commercial *M. hyopneumoniae* bacterin at arrival into the sow herds. Moreover, all suckling pigs in each flow received a dose of commercial *M. hyopneumoniae* bacterin at three weeks of age. Pigs were humanely cared for and adequate housing and nutritional demands were met or exceeded. All health and welfare standards were approved by the attending veterinarian(s).

6.3.2 Study design

A total of 262 samples, consisting of either laryngeal or bronchial swabs, were collected from the four production flows. In each flow, swabs were obtained from 1-4 herds per production stage (i.e., GDU, sow herd, nursery, and finisher) for a period of four months up to three years. Flow sample sizes were estimated to detect at least one test-positive individual in the sample with a group sensitivity of 95%. Thus, at least one VNTR type per production stage for each flow was estimated to be detected. Laryngeal swabs were obtained from pigs expressing clinical signs suggestive of *M. hyopneumoniae* infection. In the case that clinical signs were not observed, pigs to be sampled were randomly selected from the population. Bronchial swabs were collected if mortalities were observed.

6.3.3 Sample collection and processing

Laryngeal and bronchial swabs were collected using a sterile collection swab (BBL™ CultureSwab™, Becton, Dickinson and Company, Sparks, MD, USA) as previously described (Sievers et al., 2015; Pieters et al., 2017). Samples were refrigerated immediately after collection and were stored at approximately -20^{o}C until processed.

DNA extraction (MagMAX^{TM}96 Viral RNA Isolation Kit and MagMAX^{TM} Express96 Magnetic Particle Processor, Life Technologies, Grand Island, NY, USA) and species-specific *M. hyopneumoniae* real-time PCR (VetMAX^{TM} qPCR Master Mix and VetMAX^{TM} *M. hypopneumoniae* Reagents Kit, Life Technologies, Grand Island, NY, USA) were performed at the University of Minnesota Veterinary Diagnostic Laboratory. Samples with a Ct value ≤37 were considered positive for *M. hyopneumoniae* and those with a Ct value ≤32 were selected for genetic typing (Dos Santos, personal communication). To assess the genetic variability of *M. hyopneumoniae,* VNTR typing was performed as described (Dos Santos et al., 2015). The *Mycoplasma hyopneumoniae* ATCC 25095 reference strain and molecular grade water were used as a positive and negative control.

6.3.4 Data analysis

Results were analyzed using a bioinformatics analytic software (BioNumerics, version 7.1, Applied Maths, Austin, TX, USA) according to the parameters described by Dos Santos et al. (2015). A minimum spanning tree (MST) was created to illustrate the relationship between VNTR types within and across flows.

6.4 Results

Overall, 45.4% (119/262) samples were positive for *M. hyopneumoniae* by real-time PCR. Within two flows (B and D), a higher proportion of bronchial swabs (100-88.9%) were positive compared to laryngeal swabs (55.5-46.7%). A VNTR type was obtained in 83.6% (41/49) of the samples in which MLVA was attempted. A graphic representation of VNTR types from all flows over time is presented in Figure 6.1. Across

the four flows, a total of 15 VNTR types were identified, in which a single flow consisted of 1-6 VNTR types. During a single sampling event, up to three VNTR types were identified within a herd. A dominant type (i.e., 16-24, 9-12, 11-21, 13-17) was observed in flows A-D, respectively (Figure 6.1). In flows A, B, and C an identical VNTR type was identified in the GDU and grower sites (i.e., nursery or finisher) from four months up to two years (Figure 2). In addition, an identical VNTR type was identified in two finishers in flow D, for approximately two years (Figure 6.2). Based on sample type, an identical VNTR type (9-12 and 11-22) was detected in laryngeal and bronchial swabs for flows B and C, respectively. In flow D, two VNTR types (14-18 and 13-19) were only identified in bronchial swabs.

6.5 Discussion

In this study, 3-6 VNTR types were identified in three of the four flows, in which the predominant type(s) varied by 1-3 tandem repeats. Previous literature has shown the detection of multiple *M. hyopneumoniae* VNTR types in a site and group of pigs (Pantoja et al., 2016; Michiels et al., 2017). Moreover, the number and diversity of VNTR types present in a herd may be potential factors towards disease outcome as recent work has shown that lung severity may be influenced by the existence of more than one VNTR type in a herd (Vranckx et al., 2011; Michiels et al., 2017). However, the etiologic cause and importance of genomic modifications evidenced in VNTR length differences by MLVA typing has not been fully described. Since these flows were similarly managed, the potential impact of swine management and control methods, namely co-sourcing flows and different vaccine and antimicrobial programs, on *M. hyopneumoniae* genetic

variation could not be investigated. Therefore, further information is needed on this topic to help explore potential drivers of genetic diversity.

An identical VNTR type was identified over several years and production stages within a flow, which is comparable to results of Rebaque et al. (2018). In each flow, the VNTR types identified in nursery and finisher herds appeared to be derived from the breeding herds (i.e., GDU and sow herds), in which those pigs were sourced from. This statement is further supported by the detection of flow specific types, since a common VNTR type was not detected between flows. In endemic populations, the circulation of *M. hyopneumoniae* in swine production flows has been thought to originate from incoming gilts and shedding sows through dam-to-piglet transmission (Fano et al., 2007; Maes et al., 2018). Lateral transmission of this microorganism has been suggested to occur at long distances (Goodwin, 1985; Otake et al., 2010). However, recent field data proposes that the likelihood of *M. hyopneumoniae* lateral transmission in high density swine populations may be minimal (Yeske, 2017). With this knowledge, the origin of *M. hyopneumoniae* infection and VNTR type in grower-finisher sites appeared to be dependent on the sourcing herd regardless of sampling time. Such information is important to consider when structuring management of flows and *M. hyopneumoniae* control and acclimation strategies.

6.6 Conclusions

In conclusion, *M. hyopneumoniae* genetic variability within swine flows was minimal over time and flow specific under the conditions of this study. The circulation of existing *M. hyopneumoniae* VNTR types appeared to be derived from breeding herds, potentially through dam-to-piglet transmission. Further research focused on determining

the effect of different management strategies (e.g., vaccination, antimicrobials) and geographical location on *M. hyopneumoniae* genetic variability should be implemented to provide additional insight.

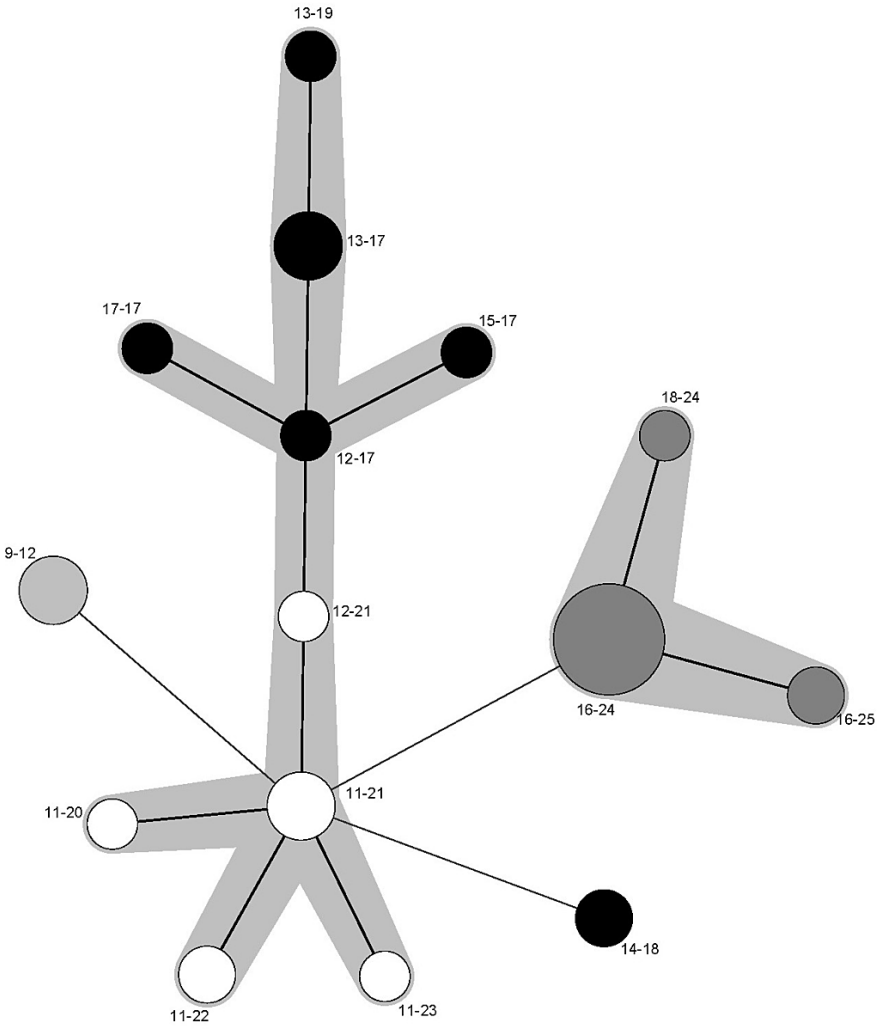

Figure 6.1 Minimum spanning tree of *Mycoplasma hyopneumoniae* Variable Number Tandem Repeat (VNTR) types

=Flow A. =Flow B. =Flow C. =Flow D.

Grey shading illustrates clonal complex of VNTR types. The lines connecting two circles show a relationship between the VNTR types, whereas the darker lines represent a clonal complex. The two numbers outside of each circle correspond to the VNTR type in the sample. Each number represents the number of tandem repeats for P97 and P146 loci, respectively. Circle size corresponds to sample size (the larger the circle, the more samples with the specific VNTR type). For example, n=1, 2, 4, and 15 for VNTR types 11-23, 11-22, 13-17, and 16-24, respectively.

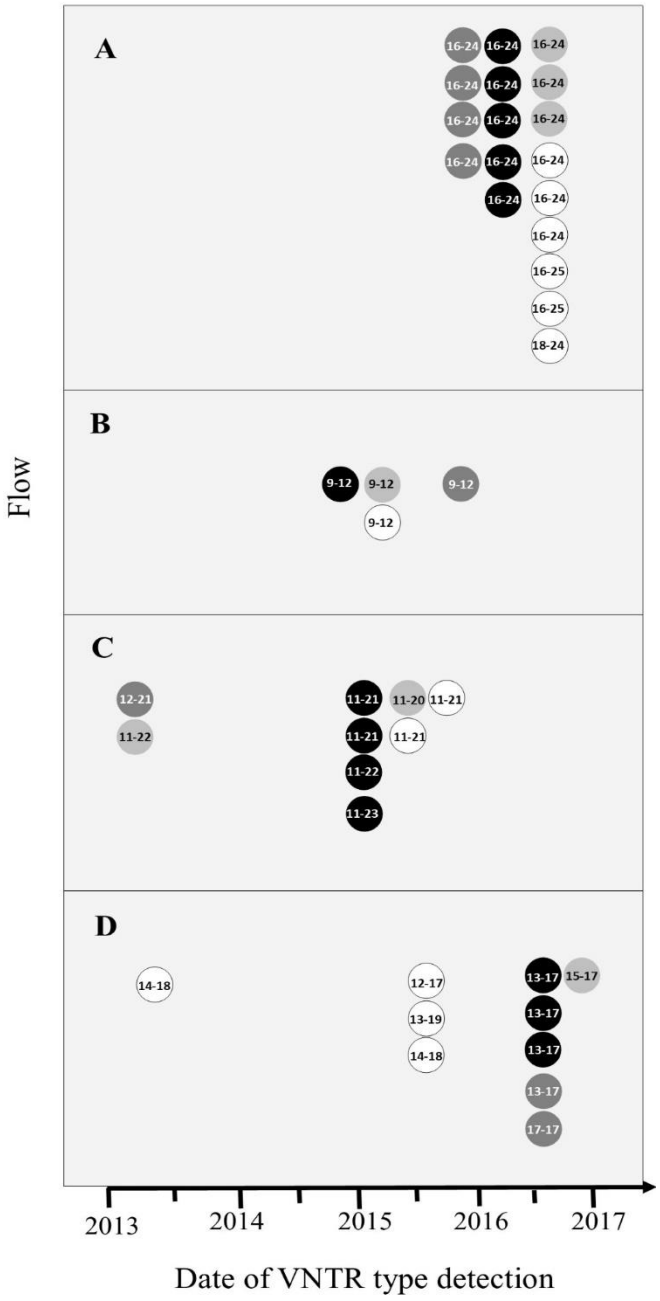

Figure 6.2 *Mycoplasma hyopneumoniae* Variable Number Tandem Repeat (VNTR) types identified over time by flow and production stage

●=Gilt developing unit. ●=Sow herd. ●=Nursery. ○=Finisher.

Each box corresponds to a production flow (A-D). Each circle represents a single VNTR type detected at a given herd and date. Within each circle, the first and second number correspond to the number of tandem repeats identified in P97 and P146 loci, respectively.

General discussion and conclusions

In the literature, ‘disease control’ has been defined as the reduction in incidence, prevalence, morbidity, or mortality of an infectious disease to a locally acceptable level, whereas ‘elimination’ is often defined as the absence of disease or infection incidence caused by a specific pathogen in a localized geographical area (Dowdle, 1998). It is important to note that disease control and elimination result from deliberate efforts that halt pathogen transmission. Furthermore, continued measures to either maintain a reduced level of infection and/or disease, or prevent re-establishment of transmission are required. Principles in achieving disease control and elimination include: 1) practical diagnostic tools with sufficient sensitivity and specificity to detect infection levels that can lead to transmission; 2) effective intervention(s) to interrupt transmission of an agent; 3) resources to trace and monitor pathogen spread; and 4) confirmation of pathogen absence in the population (Miller et al., 2006).

Over the past decade, there has been an increased interest to procure and maintain *M. hyopneumoniae* negative herds, due to the growing awareness of the detrimental and long-term effects that enzootic pneumonia has on pig health and productivity. Moreover, significant economic losses attributed to *M. hyopneumoniae* have been calculated in affected herds, thus demonstrating the value of freedom of disease. Even after the combined use of several preventive or treatment interventions, in many cases only partial disease control is achieved. Therefore, *M. hyopneumoniae* elimination is considered the only option for total disease control. In the field, the elimination of *M. hyopneumoniae*, followed by a short return of investment has been described, which makes this control method even more appealing to producers and veterinarians. However, there are several cases in which the continuation or occurrence of *M. hyopneumoniae* infection or disease

has been described, thus questioning the efficacy of control, elimination, and prevention strategies, as well as pathogen origin.

For *M. hyopneumoniae,* current knowledge of pathogen transmissibility has stemmed from studies under experimental conditions that have traditionally used clinical samples of low or medium diagnostic sensitivity. Therefore, the ability to accurately detect and predict pathogen spread may be difficult to extrapolate to field conditions. Chapter 2 of this thesis evaluated the natural transmission and detection of *M. hyopneumoniae* based on the introduction of one infected gilt to a naïve population. The ability to detect a recent *M. hyopneumoniae* introduction using clinical samples of high diagnostic sensitivity was also explored. From this study, the slow transmission of *M. hyopneumoniae* was evident, as infections derived from the index case were initially detected six weeks after introducing the infected pig. Moreover, the study's findings shed light on the potential false diagnostic assurance that can result from currently employed surveillance protocols for *M. hyopneumoniae.* It is important to mention that differences in pathogenicity and virulence have been described across *M. hyopneumoniae* strains, which can influence infection dynamics and transmissibility. Information generated in Chapter 2 can be used to design accurate surveillance protocols that are specifically tailored for *M. hyopneumoniae* in replacement gilt populations, along with the potential modification of gilt isolation procedures. During the development of surveillance protocols, a budget analysis with the inclusion of sample pooling to aid in feasibility and practicality should be conducted, as well as a long-term benefit-cost analysis that considers the economics attributed to disease prevention versus the cost of undergoing disease elimination. Secondly, transmission parameters that were calculated in Chapter 2

can be incorporated into future epidemiological models to either 1) provide insight retrospectively for the presumed onset of *M. hyopneumoniae* introduction in outbreak scenarios based on pathogen prevalence and timing of clinical disease in the herd or 2) be utilized as a prior for *M. hyopneumoniae* transmission in disease modeling.

Understanding the efficacy of interventions to slow or stop pathogen transmission is vital for optimizing *M. hyopneumoniae* control and elimination strategies. Despite their wide use, there is limited information on whether vaccinations and medication can reduce pathogen spread. In Chapter 3, the employment of multiple vaccinations on *M. hyopneumoniae* transmission was evaluated using an *in-vivo* seeder-to-contact challenge model. Results suggested that a three-dose vaccination strategy against *M. hyopneumoniae* numerically reduced the transmission of this microorganism, favoring conditions when both seeders and their corresponding contact gilts were vaccinated. Moreover, Chapter 4 explored the effect of a bacteriostatic antibiotic treatment on the infectious potential of *M. hyopneumoniae* during different phases of infection. In the fourth chapter, the administration of antibiotics during the acute phase of infection numerically reduced the rate of secondary *M. hyopneumoniae* infections. However, the role of medication on *M. hyopneumoniae* transmission could not be assessed during the chronic phase of infection. Interestingly, the persistence of *M. hyopneumoniae* genetic material detected by PCR was shown by the lack of clearance in most treated and chronically infected gilts, which lasted for up to three months, which was the last time when it was evaluated. Since the infectious potential of *M. hyopneumoniae* could not be inferred during the chronic phase of infection, research that investigates drivers of

pathogen persistence, as well as alternative methods to assess pathogen viability post-medication is needed.

The field application of results from Chapters 3 and 4 would be most beneficial for disease elimination efforts in endemically infected herds or outbreak scenarios. For *M. hyopneumoniae* elimination strategies, sow herds are either vaccinated once or quarterly during whole herd medication, or herd closure and medication programs, respectively. Furthermore, antibiotics are often administered during the last seven to four weeks of a 34-week extended herd closure and medication program. Based on the findings in Chapters 3 and 4, incorporating a multi-dose vaccination strategy or medication during the early stages of an elimination program warrants investigation, as the timing of these control interventions may influence pathogen transmissibility, resulting in potential differences in the success of elimination programs.

Another research area of interest is the effect of vaccination and medication timing on *M. hyopneumoniae* disease control and transmission during disease outbreaks. Currently, there is minimal information on this topic, and veterinarians often question which control measures should be employed and their frequency of application. Throughout the literature, several vaccination strategies, which vary based on the number or frequency of vaccinations or bacterin applications, have been explored, in which complete protection is not achieved. However, the role of vaccination timing on immune induction and whether this may aid in the reduction of pathogen spread has not been previously investigated. To provide insight on this topic, information generated in Chapters 3 and 4 can be incorporated into epidemiological models to simulate and

compare potential differences in the spread of *M. hyopneumoniae* based on medication and vaccination timing.

For the last two chapters of this dissertation, insight into the molecular epidemiology of *M. hyopneumoniae* was gained. In Chapter 5, two different molecular techniques, namely MLVA and P146 sequencing, were compared in a parallel manner to characterize circulating *M. hyopneumoniae* variants in the Midwestern United States. Findings from this study highlighted slight differences in assay sensitivity and discriminatory power between the two molecular techniques. Nevertheless, both techniques showed wide genetic diversity among *M. hyopneumoniae* variants, and similar epidemiological inferences were obtained from the two assays, as production flow explained most of the variation in the clustering of VNTR types and P146 sequences. One question that remains unanswered is the classification of *M. hyopneumoniae* variants and the biological importance of such genetic variability. Additional work utilizing whole-genome sequencing should be conducted to evaluate the presence of other variable areas within the genome that are unrepresented by MLVA and P146 sequencing. For other bacterial pathogens, whole-genome sequencing is commonly employed, largely due to feasibility in bacterial culture. Given the restrictions with *M. hyopneumoniae* isolation, development of procedures to optimize sample quality for whole-genome sequencing using clinical samples is warranted. Moreover, an ongoing database for *M. hyopneumoniae* VNTR types and/or sequences should be established, such that knowledge regarding the temporality of *M. hyopneumoniae* variants and potential bacterial evolution can be discovered. Based on the findings from Chapter 5, the genetic diversity of *M. hyopneumoniae* across swine production flows was evaluated in Chapter

6. From Chapter 6, insight for pathogen origin in downstream herds was provided based on the presence of a common *M. hyopneumoniae* variant that was shared between finishers and the sourcing herds (i.e., sow herds). Therefore, the findings from Chapter 6 showed how molecular characterization techniques could be applied in the field to help link pathogen transmission between herds.

This dissertation addressed several questions surrounding the detection, control, and molecular characterization of *M. hyopneumoniae*. The research conducted generated new information that can be readily applied in the field to aid pathogen surveillance, control, and elimination efforts.

The work presented in this dissertation also highlighted several areas that would benefit from further research such as:

- Development and economic analysis of surveillance protocols for *M. hyopneumoniae* detection in replacement gilt populations. Based on the knowledge generated in this thesis, surveillance protocols should include the collection of lower respiratory tract samples types. These protocols should consider different sample types, diagnostic tests, sample sizes, sample frequencies, and isolation length.

- Further evaluation of *M. hyopneumoniae* transmission using antibiotics with differing modes of action and application timing. To answer this topic, a simulation model would have to be developed based on data generated from *in-vivo* challenge models that evaluate differences in

transmission parameters, such as adjusted reproductive number.

- Efficacy of *M. hyopneumoniae* elimination based on strategies that vary based on timing of whole herd medication and/or vaccination. To answer this question, prospective quasi-experiments can be conducted, in which reduction in prevalence or incidence can be measured post-intervention followed by differences in length of closure based on employed strategy.

- Development of diagnostic techniques to assess pathogen viability post-antibiotic treatment. Alternative methods to evaluate pathogen viability from antemortem samples should be developed, considering limitations with using *in-vivo* models and the slow transmission of *M. hyopneumoniae.*

- Further evaluation of *M. hyopneumoniae* characterization using whole genome sequencing, along with the potential drivers for *M. hyopneumoniae* genetic diversity

In summary, an overall review of the results of this thesis was presented in this section, along with the most important implications for field applications and research questions derived from the newly generated information. In addition, future research directions were listed, which can represent the starting point of continued investigations with the goal to improve *M. hyopneumoniae* disease control and elimination.

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Appendix

Supplementary files for Chapter 3

Table S1. Diagnostic results for *Mycoplasma hyopneumoniae* detection and seroconversion

Pig ID	Treatment	Dpi	-42	-28	-14	0		28		35		42	
		Dpc	-70	-56	-42			0		7		14	
		Assay	ELISA	ELISA	ELISA	PCR	ELISA	PCR	ELISA	PCR	ELISA	PCR	PCR*	ELISA
1	NVS		71.52	83.61	88.71	-	92.86	**25.34**	**41.37**	**23.60**	**19.42**	**33.59**	**29.22**	**25.65**
2	NVC		90.49	79.45	99.14			-	98.88	-	82.24	-	-	85.30
3	NVS		85.66	90.87	84.83	-	95.51	**24.10**	54.56	**26.66**	**22.42**	**25.01**	**25.09**	**23.99**
4	NVC		83.06	85.55	86.33			-	93.63	-	92.71	-	-	85.25
5	NVS		63.10	87.33	89.53	-	97.39	**30.91**	30.63	**27.62**	**30.63**	**33.35**	**26.91**	**14.67**
6	NVC		67.70	83.33	94.28			-	90.14	-	90.20	**33.15**	**29.49**	92.64
7	NVS		81.67	91.48	91.04	-	95.90	**26.59**	42.99	**26.35**	**32.98**	**28.25**	**29.32**	**39.30**
8	NVC		80.45	91.82	86.33			-	103.99	-	79.37	-	-	92.45
9	VS		76.13	52.84	**15.34**	-	**9.26**	**20.88**	**1.29**	**33.54**	**1.74**	**34.28**	**26.31**	**1.91**
10	NVC		88.66	73.69	93.42			-	87.22	-	97.26	-	-	94.49
11	VS		87.33	62.66	**24.74**	-	**13.46**	**22.99**	**1.16**	**25.13**	**1.29**	-	**24.75**	101.26
12	NVC		92.48	85.28	88.66			-	113.58	-	89.13	**23.45**	**36.52**	**1.72**
13	VS		83.39	82.78	**14.57**	-	**4.40**	-	**8.22**	-	**13.21**	-	**38.20**	**25.49**
14	NVC		72.47	72.35	84.05			-	87.74	-	92.66	-	**38.01**	103.04
15	VS		75.07	71.08	N/A	-	**18.71**	-	**30.24**	-	**46.56**	**31.06**	**39.99**	63.16
16	NVC		97.36	80.73	78.07			-	94.22	-	92.83	-	-	97.30
17	NVS		82.28	96.14	89.82	-	99.53	**23.32**	**45.91**	**29.69**	**41.50**	**29.59**	**23.17**	**46.51**
18	VC		96.37	52.82	**14.18**			-	**14.89**	-	**17.93**	-	-	**29.73**
19	NVS		87.27	83.84	84.55	-	96.22	**20.52**	**22.21**	**26.65**	**19.68**	**26.83**	**31.40**	**21.24**
20	VC		82.56	70.19	**31.27**			-	**32.13**	-	**29.72**	-	-	**58.89**
21	NVS		103.24	84.55	79.84	-	96.09	**29.76**	**30.69**	**33.60**	**32.44**	**33.69**	**31.92**	**33.69**
22	VC		76.85	75.29	**20.39**			-	**41.12**	-	**40.73**	-	-	**64.89**
23	NVS		86.49	82.06	87.88	-	98.55	**28.85**	**45.00**	**25.45**	**34.83**	**30.09**	**25.57**	**42.36**

24	VC	79.18	60.94	**31.53**			**-**	**13.59**	**37.18**	**23.32**	**33.32**	**35.13**	**38.47**
25	VS	97.25	74.18	**17.93**	-	**2.84**	**30.39**	**3.95**	**32.87**	**5.05**	**34.29**	**21.45**	**6.63**
26	VC	72.69	66.14	**25.38**			**-**	**27.26**	**-**	**40.06**	**-**	**-**	**41.02**
27	VS	110.62	51.28	**15.28**	-	N/A	**29.56**	**1.94**	**32.03**	**2.52**	**34.34**	**29.17**	**4.75**
28	VC	59.82	75.74	**30.04**			**-**	**49.66**	**-**	**55.83**	**-**	**-**	**72.16**
29	VS	72.07	86.38	**44.09**	-	N/A	**23.58**	**2.72**	**28.27**	**3.23**	**27.44**	**25.60**	**3.57**
30	VC	71.30	66.98	**24.74**			**-**	**14.44**	**-**	**23.67**	**-**	**-**	**45.36**
31	VS	75.68	80.95	**33.15**	-	**7.64**	**30.39**	**3.69**	**33.82**	**4.72**	**38.40**	**28.68**	**8.55**
32	VC	100.91	69.58	**13.10**			**-**	**10.94**	-	**23.83**	-	**-**	**22.71**

Treatment: VS (Vaccinated seeder); NVS (Non-vaccinated seeder); VC (Vaccinated contact); NVC (Non-vaccinated contact). Dpi=days post-inoculation. Dpc=days post-contact. Positive results are bolded. Negative PCR results are indicated as (-). PCR*=Detection in bronchial swabs. ELISA results are expressed as percent inhibition and interpretated as positive for values ≤50 and negative for values >50. PCR results are expressed as Ct values and interpreted as positive for values <40.